

**Ichthyophthiriasis in fish: genetic variation in  
resistance to infection.**

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A tributary off from the Vera Cruz lake, Mexico, collection site of *Xiphophorus maculatus* (Vera Cruz). Photograph reproduced with permission from Dennis Barrett.

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## Acknowledgements.

Almost without exception, every thesis contains an acknowledgement page, through which the author attempts to convey recognition to others involved in the production of that work. I find it a grossly inadequate medium, which is often forgotten as quickly as it is read. By saying as much it is my hope that the acknowledgements below will be both emphasized and remembered.

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To all at Polytechnic South West ;

*So long and thanks for all the fish !, D. Adams. (1984)*

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## Abstract

Ichthyophthiriasis in fish: genetic variation in resistance to infection.

Graham Maurice Clayton, B.Sc., Dip. Ed.

The frequency and severity of fish diseases is, increasingly, being reported as a limiting factor in the future development of aquaculture. The control of fish disease is largely performed in retrospect through curative chemotherapy. However, the development of resistant strains of fish places the emphasis on preventative, rather than the curative, control of disease.

The freshwater parasite *Ichthyophthirius multifiliis* causes the disease known as ichthyophthiriasis or white spot. Losses of fish due to this parasite are believed to total over one million dollars per annum worldwide. The objective of this study was to examine the genetics of resistance to *I. multifiliis*.

Comparisons were made between four stocks of the tropical livebearing fish *Xiphophorus maculatus*. One of these stocks, the blue platyfish, was found to be less susceptible to white spot than the yellow comet tail, red or red wag tail platyfish. Comparisons with four other tropical species of fish found significant differences between *X. maculatus*, *X. variatus*, *Ameioba splendens* and *Ilyodon xanthusi* in levels of susceptibility. *A. splendens* was the most susceptible species, with the blue platy (*X. maculatus*) and sunset platy (*X. variatus*) forming the most resistant group. All the remaining stocks and species formed an intermediate group. Examination of resistance to white spot infection in four scale types of related common carp (*Cyprinus auratus*) also found variation in resistance, with the fully scaled carp being the most resistant phenotype (scattered mirror, linear mirror and leather carp being similar in infection level).

More detailed analysis of the genetics of disease resistance was performed with heritability determinations in stocks of *A. splendens*, *X. maculatus* (yellow comet tail) and *X. maculatus* (Vera Cruz). The highest heritability value, based on sire components only, was that for Vera Cruz platyfish of 0.75, with a value of 0.23 for *X. maculatus* (yellow comet tail) and 0.00 for *A. splendens*. A breeding programme was also performed between *X. maculatus* (red platy) and *X. variatus* (sunset platy) to evaluate the presence of any heterosis. Such was observed, with a heterosis value, based on actual parasite counts, of 16.2%.

Several factors of the infection process are also discussed, especially the fluctuating yearly trends in infection levels and parasite strain differences. Finally, the future potential of genetic manipulation of fish stocks for increased disease resistance is discussed in the light of this study. It is considered that a useful foundation has been laid for the further development of this approach to disease prevention in aquaculture.



# Chapter 1

## Introduction.

### 1.1. Problems in the future development of aquaculture.

The development of the aquaculture industry has often been compared to the development that took place over past years in the animal production industry. Perhaps the most common analogy is that drawn between aquaculture and the more recent developments of this century, in the poultry industry (Herman, 1970; Gratzek, 1983). Gjedrem (1975) suggested that the future competitiveness of fish farming, compared to other animal production, depended upon six factors; i) food conversion rates; ii) food costs and the amount available; iii) composition of the ration used; iv) frequency of fish diseases and how effectively they can be cured; v) price of products and their marketing; vi) productivity of the animals. Indeed, these six points also form a good summary of the current research areas in aquaculture. Gjedrem (1975, 1983) discussed the possible extent of genetic gain, as defined by Falconer (1981), for the five traits which he considered as of greatest importance to the development of fish farming, namely; i) growth rate from hatching to harvesting; ii) food conversion; iii) viability and resistance to disease; iv) carcass quality; v) age at sexual maturity. Kirpichnikov (1971b) presented a very similar list of principal aims to be accomplished in future fish selection programmes. The problems with fish disease have, increasingly, been reported as limiting to the full exploitation of fish farming (Herman, 1970; Meyer & Hoffman, 1976; Price & Bone, 1985; Ocvirk, 1987). This investigation is concerned solely with the area of disease resistance in fish and its' contribution to a more productive and efficient aquacultural industry.

## 1.2. Fish disease in aquaculture.

It is not surprising that fish disease epizootics, whilst not unknown (Allison & Kelley, 1963; Meyer & Hoffman, 1976) are relatively uncommon in wild stocks of fish. However, when fish are farmed they are placed into a much more demanding environment (Herman, 1970). They are crowded, handled and largely dependant upon the farmer for their food ration. As a consequence disease is much more prevalent and the consequences of that disease often much more drastic. The cost of disease to the farmer can be huge, not only in terms of lost stock, but also in terms of treatment and reduced food conversion of healthy stock (Ocvirk & Bravnicar, 1984, 1987).

Brown & Gratzek (1980) estimated the on-farm value of cultured freshwater species in the USA alone to be \$200-300 million. Further figures for the value of cultured species are sparse. Much of the information available on this subject relates to wild populations which are exported as ornamental species. Such figures, however, are useful as an indication of the impact of disease upon undomesticated stocks. Conroy *et al* (1981) quote 1973 exportation values of ornamental species to the USA as \$832,519, \$438,557 and \$68,448 for Colombia, Peru and Venezuela respectively. In 1972 the USA retail trade in ornamental fish alone was estimated as \$250 million. (Conroy, 1975), and imports into the USA of ornamental species from worldwide sources in 1973 amounted to \$8,853,430 (Conroy *et al*, 1981).

The impact that fish disease can have on these figures can be difficult to estimate. Klontz (1985) reports estimates of 10-30% of production cost is lost through disease. Conroy (1975) and Conroy *et al* (1981) gave a figure for total losses of ornamental fish, from all causes, prior to exportation from Peru as 55.2%. This amounts to \$242,083 for fish exported in 1973 from Peru, to the USA alone. Similar figures for Colombia (Vásquez, 1974) indicate up to \$499,511 (ie. 60%) is lost through death of fish prior to exportation to the USA. Shepherd (1987) suggested a reduction in return on investment from 39% to 21% might occur from a recurring furunculosis problem, over the two year production cycle on a 250 metric ton salmon farm. This is equivalent to a £70,000 reduction in pre-tax profits.

To date there are more than twenty cutaneous and systemic bacterial diseases , more than thirty viral diseases and more than one hundred external and internal parasitic diseases of commercially important fish species (Ellis, 1987). These diseases can result in a reduced fitness, and hence productivity of stock, lost sales, the cost of purchasing chemotherapeutants, or, in severe cases, the cost of replacing the complete stock. Therefore, there is an urgent need to improve the control of fish disease in aquaculture in order to increase the efficiency and viability of fish farms.

McGregor (1963) reviewed the literature on fish parasites and diseases from 330 B.C. - 1923 A.D. The study of disease in the culture of fish is, therefore, a very old science. However, much of the early study was purely descriptive of the disease symptoms. Where the causative agent could be observed, which usually meant the parasite, then taxonomical studies were performed. In the last decade of the 19<sup>th</sup> Century bacterial diseases were first described. Extensive problems in the early 1900's lead to the commission by the British Government of the Furunculosis Committee in 1930. The committee, which existed for five years, did much to further the study of many diseases, and not just furunculosis. This, perhaps, paved the way for the future explosive growth in fish farming in the 1960's and 1970's. To-day there are many fish pathologists, and an increasing number of courses which include some fish pathology training. Associations such as the European Association of Fish Pathologists exist and at least one journal is entirely devoted to fish pathology (*ie.* Journal of Fish Disease). Much money, time and effort now goes into the more effective and efficient control of fish disease.

### **1.3. Control of fish disease.**

The control of fish disease has many facets and can be divided into four categories; i) improved husbandry; ii) chemotherapy; iii) vaccination; iv) breeding for resistance.

### **1.3.1. Improved husbandry.**

Improving the husbandry practices has two distinct advantages. The first is to at least assist in the control of a disease outbreak by providing the optimum conditions for the fish to recover. In addition, future outbreaks and transmission of disease to other ponds can be prevented. Munro & Fijan (1981) reviewed the techniques in controlling and preventing disease on fish farms. Water quality and condition is perhaps the prime concern. Often a drop in water quality can be the trigger for a disease outbreak. The introduction of further pathogens can also be stopped by attention to the foodstuffs, quarantine procedure, water supply and fish/egg sources. In addition disinfection and sterilization procedures are important in control and prevention. Improved husbandry is, therefore, very simple in many ways, but is also often overlooked, resulting in recurring and spreading disease outbreaks.

### **1.3.2. Chemotherapy.**

Chemotherapy is, undoubtedly, the major method of control of fish disease. There is an enormous range of chemotherapeutants available, and the list grows continually, as new and more effective drugs are developed. The addition of natural and semi-synthetic antibiotics to this list merely serves to provide a multitude of possible cures for a disease. Herman (1970), Herwig (1979), Snieszko (1978), Gratzek (1983), and Austin (1984, 1985) provide summaries of the common antimicrobials (*ie.* chemotherapeutants and antibiotics) in use. Reichenbach-Klinke & Elkan (1965) and Meyer and Hoffman (1976) provide two of the perhaps more well-known and generally more available discussions of the treatment of fish disease. The number of such textbooks has however grown dramatically over the past few years (*e.g.* Snieszko & Axelrod, 1971; Snieszko, 1980; Palmer & McArdle, 1982; Roberts, 1978, 1982; Post, 1983).

There are several problems in the use of chemotherapy to control disease outbreaks (Meyer & Hoffman, 1976). In some cases the size of the water body and price of the drug can make cost prohibitive (Leteux & Meyer, 1972). The

action of many chemotherapeutants is also affected by the level of organic material present (Allison, 1953). In addition, the widespread use of chemotherapeutants can lead to the production of strains of pathogens which are resistant to chemotherapy, especially among the bacteria (e.g. Herman, 1970; Aoki *et al*, 1981; Davies, 1981; Sabath, 1982). For this reason prophylactic treatment with a single antimicrobial is eventually self-defeating. The chemotherapeutant has also to be administered and this may present problems if injection is necessary. Many chemotherapeutants are also not without their secondary effects. Cross (1972) found that methylene blue adversely affected feeding habits and formalin is known to cause gill filament damage (Leteux & Meyer, 1972).

The use of medically important agents may also have dangerous consequences if resistant pathogens arose (Austin, 1985). Such resistance could well be passed onto human pathogens, with obvious consequences. Connected with this point is the problem of chemotherapeutants being discharged into river systems, where they can be mixed with the discharge from sewerage treatment works. Such pollution can also be hazardous to general river life and water supplies (Austin, 1985). Possible health hazards to the user, and shelf-life/stability of the antimicrobial, are further important considerations. Finally, antimicrobials take some time to be excreted from a fish's body and this depends upon species, temperature and antimicrobial. Such residues can be a health hazard if these fish are to be sold for the table (Gratzek, 1983 ; Austin, 1985).

### **1.3.3. Vaccination.**

The development of vaccines over the recent years provides a more promising method of control of fish disease (Gratzek, 1983). The emphasis here is more on prevention, rather than cure. Again there are problems with administration and cost, but many of the problems of chemotherapy do not exist. Pollution, withdrawal periods for sale of treated fish and the development of resistance are not of concern when vaccines are used. However, the efficiency of the

vaccine, the species it can be used with and the effect of temperature upon its effectiveness are all important variables. Field trials of vaccines are therefore often unsuccessful in demonstrating their efficiency (Baudin Laurencin & Tangtrongpiros, 1980; Smith *et al*, 1980; Michel, 1982). As a consequence only three vaccines are commercially available in the UK, all of which are for bacterial pathogens; *Vibrio anguillarum*, which causes the disease vibriosis; *Aeromonas salmonicida*, which causes the disease furunculosis; *Yersinia ruckeri*, which causes the disease enteric redmouth. Several experimental vaccines are under development, with again a heavy bias towards bacterial diseases. Very few vaccines for parasitic diseases are even at the developmental stage.

It is likely to be some time before any of the experimental vaccines are commercially available, principally due to the stringent tests required by law. Essentially this takes three stages. Firstly, the vaccine must be shown to work *in vitro*. This is followed by *in vivo* tests on laboratory animals. Finally, field trials are required to demonstrate that the vaccine will be effective under farm conditions. This whole process has to be repeated for every species of fish the vaccine is to be licensed for use with on a farm. Widespread use of vaccines for many of the common diseases is, therefore, some time off. Indeed, Ellis (1987) argued that the 'easy' vaccines have now been developed for fish. Further progress rests on future developments in the analysis of the vaccination process.

#### **1.3.4. Breeding for resistance.**

The remaining method for the control of fish disease is the development of resistant strains through selective breeding. Again the emphasis is more on prevention rather than cure. By improving the gene pool of future populations, the possibility of disease outbreaks can be reduced, since;

"A clinically manifest disease only results when proper combination of the genotype of the victim, and the genotype of the pathogen, where one is necessary, are properly synchronized with the environment." Gowen (1952).

The existence of natural resistance to infection has been known for some time

(Wolf, 1954; Snieszko, 1957, 1958; Bauer, 1971). At a meeting of the F.A.O. *Ad Hoc* Working Party on Genetic Selection and the Conservation of Genetic Resources of Fish in 1972 the development of resistant strains was recommended. The earliest work along these lines was probably that by Embury & Hayford (1925) and Hayford & Embury (1930), where brook trout were used. Price (1985) recently reviewed the work done on the genetics of fish disease.

Several diseases have been used in studies on variation in resistance/susceptibility: - bacterial kidney disease (Suzumoto *et al*, 1977), blue sac disease (Ayles, 1974), channel catfish virus (Plumb *et al*, 1975), *Chilodonella cyprini* infection (Ocvirk, 1987), dropsy (Schäperclaus, 1953, 1954; Bauer, 1971; Kirpichnikov & Factorovitch, 1972; Kirpichnikov, 1971b, 1973; Kirpichnikov *et al*, 1979), enteric redmouth (Knittel, 1981), epidermal epithelioma (Hines *et al*, 1974), fish pox (Hines *et al*, 1974), furunculosis (Embury & Hayford, 1925; Davis, 1946; Wolf, 1954; Snieszko, 1957; Snieszko *et al*, 1959; Ehlinger, 1977; Ferguson & Rice, 1980), gas bubble disease (Cramer & McIntyre, 1976), *Gyrodactylus* infection (Suzuki & Yamaguchi, 1980; Madhavi & Anderson, 1985), *Ichthyobodo necator* infection (Ocvirk, 1987), infectious haemopoietic necrosis (Amend & Nelson, 1977; McIntyre & Amend, 1978), red spot disease (Schäperclaus, 1953), salmonid blood spot (Llewellyn, 1980), ulcer disease (Wolf, 1954; Snieszko *et al*, 1959), vibriosis (Gjedrem & Aulstad, 1974; Smoker, 1981; Refstie, 1982), viral haemorrhagic septicaemia (Ord *et al*, 1976) and white spot (Bone, 1983; Price & Bone, 1985).

Certain selection programmes have increased the level of resistance to a pathogen, but the resistance is never absolute. Price (1985) commented that this may well be due to the fact that resistance is often measured on a scale several steps removed from the process of infection. Mortality over a given period of time is an oft used scale of resistance, as is the categorisation of fish into infected and not infected groups. This, unfortunate necessity, is usually the result of being unable to record infection levels in/on the fish. In some disease investigations, insufficient knowledge of the epidemiology of the disease has

precluded controlled experimental infections. Kirpichnikov *et al* (1979) also stressed that there is often an inability to exclude the possibility of death from other causes. However, Gordon (1953), Purdom (1974) and Ellis (1987) commented that, although not much advancement has been made in selective breeding for disease resistance in fish, the effective use of this approach in many other forms of agriculture suggests it is a worthwhile approach. Woo (1987) also saw selective breeding as a viable approach to protection against infection by pathogenic parasites in fish.

To allow such an approach several criteria need to be fulfilled. Ideally, occurrence of the disease on a fish would be measured on a continuous scale, to enable the level of resistance to be measured. This permits changes to be made in resistance more easily and facilitates genetic analysis. The pathogen used must preferably, therefore, be one where the etiology has been thoroughly examined. This will facilitate the controlled exposure of fish to the pathogen and accurate assessment of the infection level. The extent of genetic control of resistance must then be determined, so that a suitable approach of exploiting the genetic variation present can be derived.

#### **1.4. *Ichthyophthirius multifiliis*.**

The freshwater parasite *I. multifiliis* causes the disease known as white spot, ichthyophthiriasis or 'ich'. It is a suitable candidate to use as a pathogen, in a study on the genetics of disease resistance, since it fills all the criteria above. The extent of the losses, both in terms of stock and finance, due to *I. multifiliis* has been considered as equal to the losses from all other fish parasites combined (Hines & Spira, 1973a; Antychowicz *et al*, 1982). Migala (1971) regarded it as the most dangerous parasite in the culture of carp. In 1961 *I. multifiliis* was believed to have cost \$1,000,000 worldwide (Rahawy, cited in Allison, 1968). Herman *et al* (1959), Reichenbach-Klinke & Elkan (1965), Nigrelli *et al* (1976) and Goven *et al* (1980a) also considered it as a parasite which caused great economic losses. The etiology has been extensively studied resulting in a deep knowledge of the life cycle. Controlled infections are



possible and evaluation of the infection level is a simple matter of counting the mature parasites on the host. Discussion of the parasite in this introduction will be divided into two parts; i) The etiology and epidemiology of ichthyophthiriasis and ii) The control and treatment of ichthyophthiriasis.

#### 1.4.1. The etiology and epidemiology of ichthyophthiriasis.

*I. multifiliis* was well known in the Middle Ages (Nigrelli *et al*, 1976), but Dashu & Lien-Siang (1960) claim that the earliest description of *I. multifiliis* is to be found in a book, edited by Su-shih, sometime during the Sung Dynasty (A.D. 964-1126). Following this, the next known description is that of Hilgendorf & Paulicki (1869), from fish kept in the Zoological gardens of Hamburg, Germany. However, it was Fouquet (1876) who suggested the name of *Ichthyophthirius multifiliis*. Two early synonyms for *I. multifiliis* were *Chromatophagus parasiticus* (Kerbert, 1884) and *Ichthyophthirius cryptostomus* (Zacharias, 1892a, 1892b), but these rapidly fell out of use. According to the revised classification of the Protozoa (Levine *et al*, 1980), *I. multifiliis* is now classified as;

|                  |                           |
|------------------|---------------------------|
| <u>Phylum</u>    | : Ciliophora              |
| <u>Class</u>     | : Oligohymenophora        |
| <u>Sub-class</u> | : Hymenostomata           |
| <u>Order</u>     | : Hymenostomatida         |
| <u>Sub-order</u> | : Ophryoglenina           |
| <u>Genus</u>     | : <i>Ichthyophthirius</i> |
| <u>Species</u>   | : <i>multifiliis</i>      |

The life cycle is a simple, direct one. Mature parasites, known as trophozoites, leave the host (Figure 1) and, after a short free-swimming period, when they are known as trophonts, they settle upon a suitable substrate. Here a proteinaceous cyst wall is secreted and the parasite undergoes numerous divisions (Figure 2). After a period of time, dependent upon water temperature, the cyst ruptures and free-swimming daughter cells are released (Figure 3). The number released per cyst is generally below 1000 (MacLennan, 1935a, 1935b; Butcher, 1943; Reichenbach-Klinke & Elkan, 1965; Beckert, 1975; Nigrelli *et al*, 1976; Ewing *et al*, 1982, 1983, 1986; McCallum, 1985; McLay, 1985), although figures of upto 2000 have been reported (Prytherch, 1924;

Butcher, 1941; Meyer, 1974). Initially these daughter cells are spherical in shape (18-40  $\mu\text{m}$  in diameter, MacLennan, 1937, 1942; McLay, 1985) and are known as tomites. These tomites rapidly become elongated (length of 10-45 $\mu\text{m}$ , MacLennan, 1935b, 1936, 1937; Hines & Spira, 1973a; Meyer, 1974; Nigrelli *et al*, 1976; McCallum, 1982; McLay, 1985), from which point they are known as theronts (Figure 4). These swim in rapid, jerking movements and infect fish by burrowing into the skin, fin and gill tissue.

It is not known whether the parasite favours any particular site(s) on the host. Butcher (1941) reports that the parasite is to be found on any unscaled portion of the fish, although in a later report (Butcher, 1947) brown and rainbow trout were reported as infected over the body, head, eyes, fins, opercula and gills. Interestingly, the gills were reported as harbouring comparatively few parasites. Meyer (1974) reported that Golden shiner minnows (*Notemigonus crysoleucas*) seldom possess parasites on the body, but frequently harbour heavy infections on the gills. Parker (1965) examined *I. multifiliis* burdens upon goldfish (*Cyprinus auratus*), where only 0.3% of parasites on the fish were to be found on the gills or in the buccal cavity. The body and caudal fin carried 86.8% of the total parasite burden, but since these two regions also make up the majority of possible parasite attachment sites the significance of such a high percentage cannot be commented upon. Lucky (1970) examined 10 *Silurus glanis* specimens infected with *I. multifiliis*. Of the 130-165 parasites, 39.8% were on the lower part of the head and abdomen; 24% were on the lower part of the back, anal fin and lower part of the caudal fin; 27% were on the upper part of the body and head; and 14.5% were on the upper back and caudal fin. Hence, greater numbers were present on the more anterior, ventral surfaces. Kozel (1976), reporting on the examination of 16 blackstripe topminnows (*Fundulus notatus*), found more *I. multifiliis* on the caudal fin, head and upper body surface of its host. Hines & Spira (1973b) also commented that there appears to be more parasites on the dorsal surface of *C. carpio*. McCallum (1985) though, found no evidence that the density of parasites differed significantly on different parts of the body surface in the black molly (*Poecilia latipinna*).

The trophozoites, as they are now known, remain on the host until mature and the cycle is then complete. This stage of the parasite on the host is the source of the common name of white spot disease. As the trophozoite grows it becomes visible to the naked eye as a white spot (Figure 5), upto 1mm in diameter (MacLennan, 1935b; Butcher, 1943; Wagner, 1960; Meyer, 1974; Nigrelli *et al*, 1976). Each trophozoite arises from one theront, although there is a body of opinion that more than one parasite may be present in one 'white spot' (e.g. Stiles, 1893; Neresheimer, 1908; Prytherch, 1924; Roughley, 1933; Suzuki, 1935; Schäperclaus, 1941; Becker, 1942; Reichenbach-Klinke & Elkan, 1965; Nigrelli *et al*, 1976; Chapman, 1984). Butcher (1941) and Lucky (1970), however, examined several hundred trophozoites and were unable to support this theory, as were Haas (1933) and MacLennan (1935a). Later, Butcher (1943) did find an average of six parasites per 'white spot', but did not favour the idea of division of the parasite having occurred on the host.

The duration of the life cycle is dependant upon water temperature (Suzuki, 1935; MacLennan, 1937, 1942; Wolf, 1938; Butcher, 1941, 1943, 1947; Schäperclaus, 1954; Herman, 1958; Bauer, 1961; Cross, 1972; Cross & Hursey, 1973; van Duijn, 1973; Canella & Rocchi-Canella, 1976). At 27°C the complete cycle takes about 5 days, whilst at 10°C the life cycle can be in excess of 5 weeks. Wagner (1960), Nigrelli *et al* (1976) and Ewing *et al* (1986) also report that the number of theronts produced per cyst increases with temperature.

This simple life cycle has often been thought of as incomplete. No sexual stage is known (Haas, 1933) and there is a sizeable body of opinion that some form of dormant stage exists. Outbreaks of the disease have been known to occur in the aquarium, at this Polytechnic, when no new fish or live food had been introduced for several weeks previous. (The aquarium is run on a closed recirculation system and hence no water changes had been recently performed either.) Butcher (1943) is the only person to have tackled these variations in the standard life cycle in any great detail. Following rupture of the cyst, and the release of theronts, Butcher (1943) records that some of the theronts may settle

and divide again. He was unable to determine, though, whether the theronts which emerged following this second division were able to infect fish. The existence of latent infections has been reported by Schäperclaus (1933, 1954) and Johnson (1961), but a latent stage has not been identified.

Parker (1965) and Houghton (1987) present a comprehensive review of the published literature on *I. multifiliis*, which will not be duplicated here. Most of the early work was on structure and the life cycle of the parasite (Stiles, 1893; Haas, 1933; MacLennan, 1935a, 1935b, 1936, 1937; Mugard, 1949; Schäperclaus, 1954; Wagner, 1960; Canella & Rocchi-Canella, 1976; Ewing *et al*, 1985; Ewing & Kocan, 1986, 1987). In the mid-1960's the addition of the electron-microscope added a further body of papers on the ultrastructure of *I. multifiliis* (Mosevitch, 1965; Roque *et al*, 1967; Lom & Corliss, 1971; Hauser, 1972, 1973; Crilley & Buckelew, 1977; Chapman & Kern, 1983; Ewing *et al*, 1983, 1985; Chapman, 1984; McCartney *et al*, 1985; Ewing & Kocan, 1986, 1987). Throughout all this period there has consistently been reports, which were of a purely descriptive nature, of epizootics of this disease (Butcher, 1941, 1947; Mallié, 1946; Elser, 1955; Johnson, 1961; Allison & Kelley, 1963; Hoffman, 1967; Pozo & Roman, 1969; Lahav & Sarig, 1973; Kennedy, 1974; Khalifa *et al*, 1983; Wahli & Meier, 1987). These reports include both wild and cultivated populations of fish.

*I. multifiliis* is world wide in its' distribution, from tropical to sub-artic climates (Hoffman & Bauer, 1971). Houghton (1987) cites thirty-six separate papers on reports of *I. multifiliis* from seventeen different countries; Australia, China, Czechoslovakia, Great Britian, Finland, France, Greece, India, Iraq, Israel, Poland, South Africa, Spain, Switzerland, Uganda, USA and the USSR. This is by no means an exhaustive list, *e.g.* *I. multifiliis* has been reported from Venezuela (Conroy & Vásquez, 1976; Bermúdez, 1980; Conroy *et al*, 1981,1982). The parasite is a purely freshwater organism and infects most freshwater species of fish. Houghton (1987) lists the following as examples of species in which epizootics have been reported; the barbel (*Barbus barbus*), brown trout (*Salmo trutta*), carp (*Cyprinus carpio*), channel catfish (*Ictalurus*

*punctatus*), rainbow trout (*S. gairdneri*), salmon (*S. salar*), shad (*Dorosoma cepedianum* & *D. petenense*), Tilapia species, and yellow perch (*Perca flavens*). Numerous other species have also been reported, and it is perhaps more interesting that not every species, or individual present, is always infected (Butcher, 1941; Elser, 1955; Lahav & Sarig, 1973; Conroy & Vásquez, 1976; Baker & Crites, 1976; Kozel, 1976; Calenius, 1980; Li & Dessler, 1985; Whali & Meier, 1985). Nigrelli *et al*, (1976) stated that some fish always have a few individuals of *I. multifiliis* present, whilst some species are highly susceptible to infection. Bauer (1953) and Meyer (1974) though commented that all pond fish are susceptible to ichthyophthiriasis.

Dickerson *et al* (1981) observed erratic and low infections, but these were attributed to poor estimation of theront numbers. Reichenbach-Klinke & Elkan (1965) suggested that variation in levels of infection is possibly due to the differences in powers of resistance of the fish. McCallum (1982) also reported variation in host susceptibility and suggested genetic differences, and factors such as stress, as possible explanations. Butcher (1947) reports percentage losses of brown and rainbow trout in an ichthyophthiriasis epizootic. Over two seasons 63.75% of brown trout and 76.8% of rainbow trout were lost, possibly indicating some difference in susceptibility between these two fish species. Following the F.A.O. (1972) suggestion of the development of disease resistant strains of fish, Bone (1983) and Price & Bone (1985) studied the genetical variation in resistance to *I. multifiliis*. Significant differences between families in resistance to white spot were found in the one stock of Platyfish (*X. maculatus*, red wagtail) examined. Maternal effects accounted for 95% of the variation in resistance of these viviparous fish. It is not known whether this is the case in other stocks of fish, or whether this is peculiar to viviparous fish.

Subasinghe (1986) found juvenile *Oreochromis mossambicus* to be highly susceptible compared to older specimens, indicating a definite age dependency on susceptibility. Butcher (1947) also noted an increased susceptibility in brown and rainbow trout fry compared to yearlings. Kozel (1976), using length as an indicator of age, found more severe infestations in younger fish. In addition,

Pickering & Christie (1980) recorded a definite sexual difference in the incidence and severity of infection in the brown trout, *Salmo trutta*. Male fish were more frequently infected than immature fish of either sex, or mature female fish.

Herman *et al* (1959), Paperna (1972) and Nigrelli *et al* (1976) suggested the possibility of physiological races and species of *I. multifiliis*, which could account for some of the variation in levels of infection reported. This view was endorsed by Schäperclaus (1954), Amlacher (1961), Reichenbach-Klinke & Elkan (1965) and Hines & Spira (1973b). Other authors have also, obliquely, referred to the possible existence of strains, and sub-species, of *I. multifiliis* (Clemens & Sneed, 1958; Ghadially, 1964a; Allen & Avault, 1970; Cross, 1972). Subasinghe & Sommerville (1985) investigated the possible differences between two strains of *I. multifiliis*, which originated from distinctly different climates. No differences were observed in the reproductive capabilities of the two strains, but how far reaching this similarity extends was unknown. Hines & Spira (1973b) found no difference in virulence or pathogenicity in two isolates, although these arose from similar sources. The question of the existence of races and sub-species of *I. multifiliis* remains, therefore, unanswered.

#### **1.4.2. The control and treatment of ichthyophthiriasis.**

This is an area upon which much work has been published. Stiles (1893) was the first to suggest a chemical for the control of *I. multifiliis* outbreaks. This made use of the fact that *I. multifiliis* is a purely freshwater organism and the addition of sodium chloride would control a disease outbreak. The success was limited though. Other treatments which have been shown to work, with varying levels of success, are ; acriflavine, amphrolium, atabine, chloramine T, copper sulphate, enheptin, formalin, ironidizole, malachite green, mepacrine hydrochloride, mercurochrome, methylene blue, metronidazole, neguvon, nifurpirinol, potassium permanganate, praziquantel, pyridylmercuric acetate, quinine, silver nitrate, tramisol and trypan blue. The most effective are formalin, malachite green and methylene blue and most commercially available

formulations for controlling ichthyophthiriasis tend to be a combination of these three chemicals. None of these formulations affect parasites which are present as trophozoites on the host (Antychowicz, 1977; Antychowicz *et al*, 1982). Indeed, only one chemical, mercurous acetate, has been clearly shown to affect trophozoites. This was first reported in China by Dashu & Lien-Siang (1960). Nousias (1987) is the only person known to have tested this claim outside China. Concentrations as low as 0.25 ppm killed 100% of the parasites *in situ* following a 3h treatment. However, it is strongly recommended that mercury compounds are not used on fish destined for consumption, even at such low concentrations. Hoffman & Meyer (1974) cite three cases where trophozoites were killed with brilliant green or basic violet, but there has never been any confirmation of this by other workers. A satisfactory chemotherapeutant which acts against trophozoites is, therefore, still lacking. The continued search for more efficient chemicals in controlling ichthyophthiriasis is evidence of the unsatisfactory chemotherapeutic solution to the treatment of the disease, in all but individual aquarium situations.

Other, management techniques, have also been tried, to control and prevent disease outbreaks. Rychlicki (1968) raised the pH, using quicklime, from 7.0-7.5 to 8.5-8.9. Wagner (1960) also recorded the effect of extreme pH on the parasite. Increasing the water flow, to flush away parasites as they leave the host has also been attempted (Prytherch, 1924; Barthélémy, 1926; Maclennan, 1935b; Wolf, 1938; Allison, 1953; Bauer, 1958; Hickling, 1962; Conrad & Wyatt, 1970; Hoffman, 1970). Increasing the temperature may have some beneficial effects (Allison, 1957; Ghadially, 1964b; Reichenbach-Klinke & Elkan, 1965; van Dujin, 1973; Meyer, 1974; Leibovitz, 1980), but this is only of any real practical use for fish in aquaria. Electrotherapy was unsuccessfully tried (Farley & Heckman, 1980) and Gratzek *et al* (1983) investigated the use of ultra-violet light. This reduced levels of ichthyophthiriasis outbreaks from 82.8% to 1.3%. However, this method still has several disadvantages. In many cases cost would be prohibitive, and efficiency is greatest only in closed recirculation systems. X-rays have also been reported to affect the development of the parasite (Schrader, 1949), but no detailed study has been performed. Wahli *et*

*al* (1985) reported that increasing the level of ascorbic acid in the diet increased the survival of rainbow trout following *I. multifiliis* infection.

Bauer (1953, 1955, 1961), Beckert & Allison (1964), Hines & Spira (1973a, 1974) and McCallum (1986) demonstrated that fish could acquire immunity to infection with *I. multifiliis*. Subsequently, there has been an increasing interest in developing a vaccine against ichthyophthiriasis (Goven *et al*, 1980a, 1980b, 1981; Wolf & Markiv, 1982; Dickerson *et al*, 1984; Houghton & Matthews, 1986; Houghton, 1987). The use of *Tetrahymena* species was advocated (Dickerson *et al*, 1984), since this ciliate is easily cultured, facilitating vaccine production. However, it now appears that there is no cross-reactivity between *Tetrahymena* and *I. multifiliis* vaccines (Houghton, 1987) resulting in the need for a new approach.

### **1.5. Aims and objectives of this study.**

Given the problems with chemotherapeutic and other methods of control of white spot disease, the most promising control methods appear to be in prevention, rather than cure. This also has the advantage of producing healthier fish, which grow better than their counterparts who were able to be cured in a disease outbreak. Work on the genetics of resistance to white spot is severely lacking. In this study the principal aim was to perform a detailed study of the levels of genetic variation to ichthyophthiriasis. Inter- and intra-specific comparisons were undertaken in a variety of ways and genetic variation assessed. Attempts were made to determine the best approach by which resistance to *I. multifiliis* infection could be improved.



Figure 1 Trophont stage of *Icthyophthirius multifiliis*.

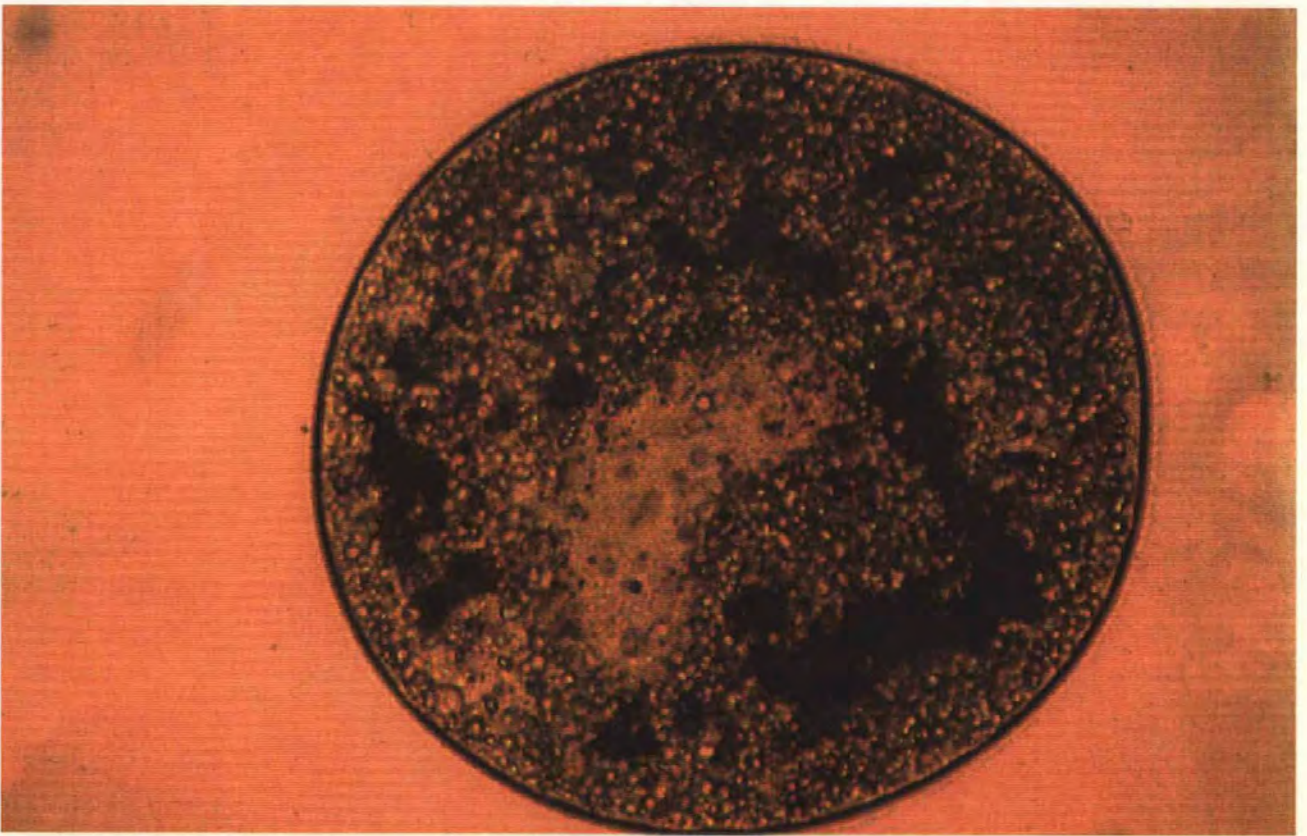


Figure 2 Cyst of *Ichthyophthirius multifiliis* in early dividing phase.

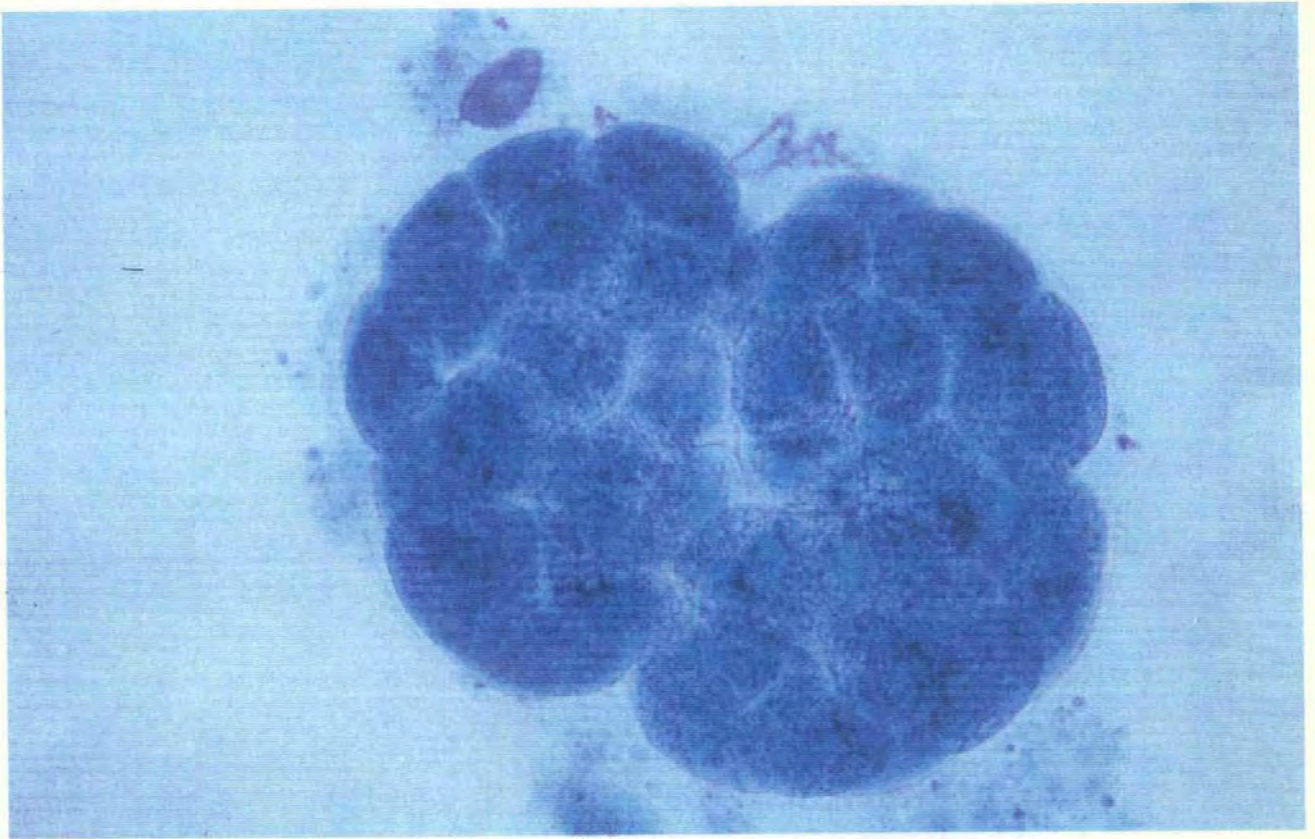


Figure 3 *Icthyophthirius multifiliis* cyst rupturing to release tomites.

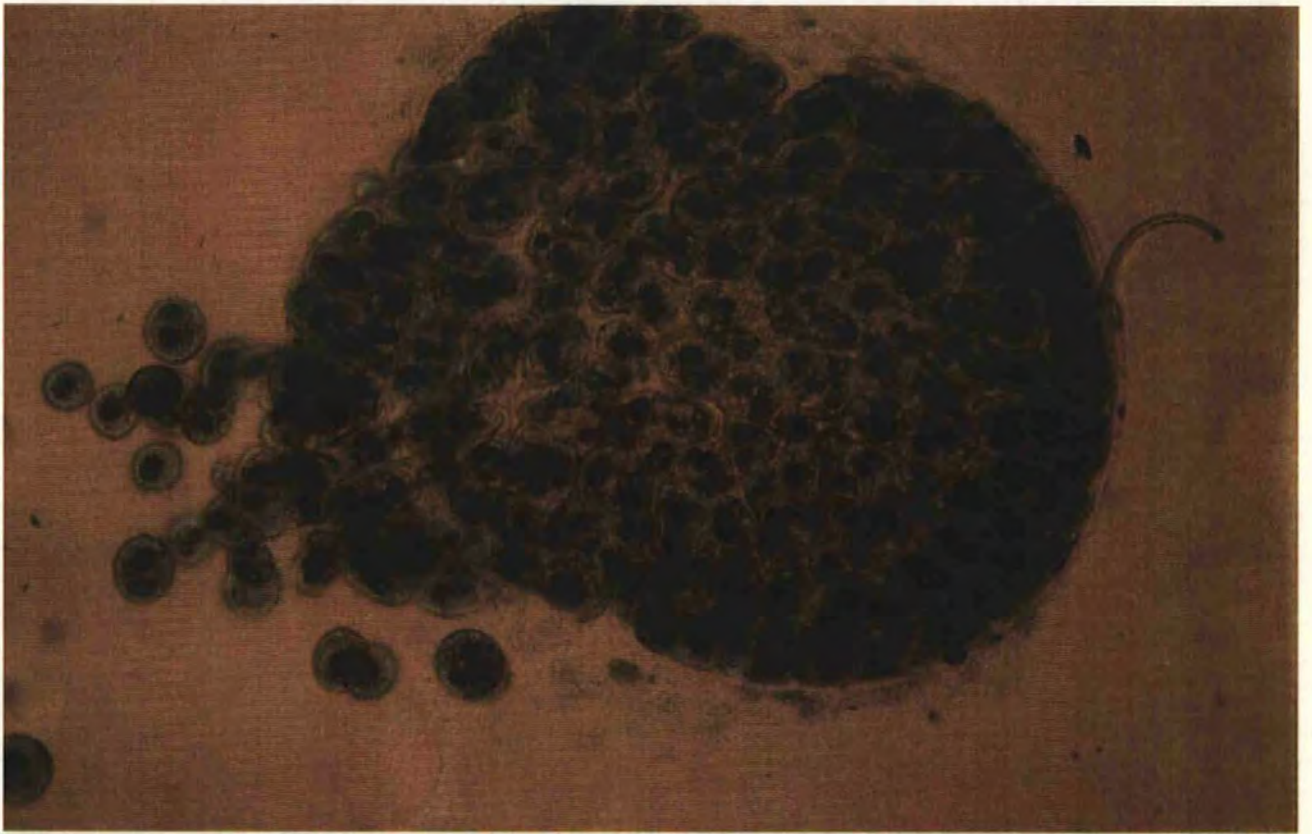


Figure 4 Theront stage of *Ichthyophthirius multifiliis*.

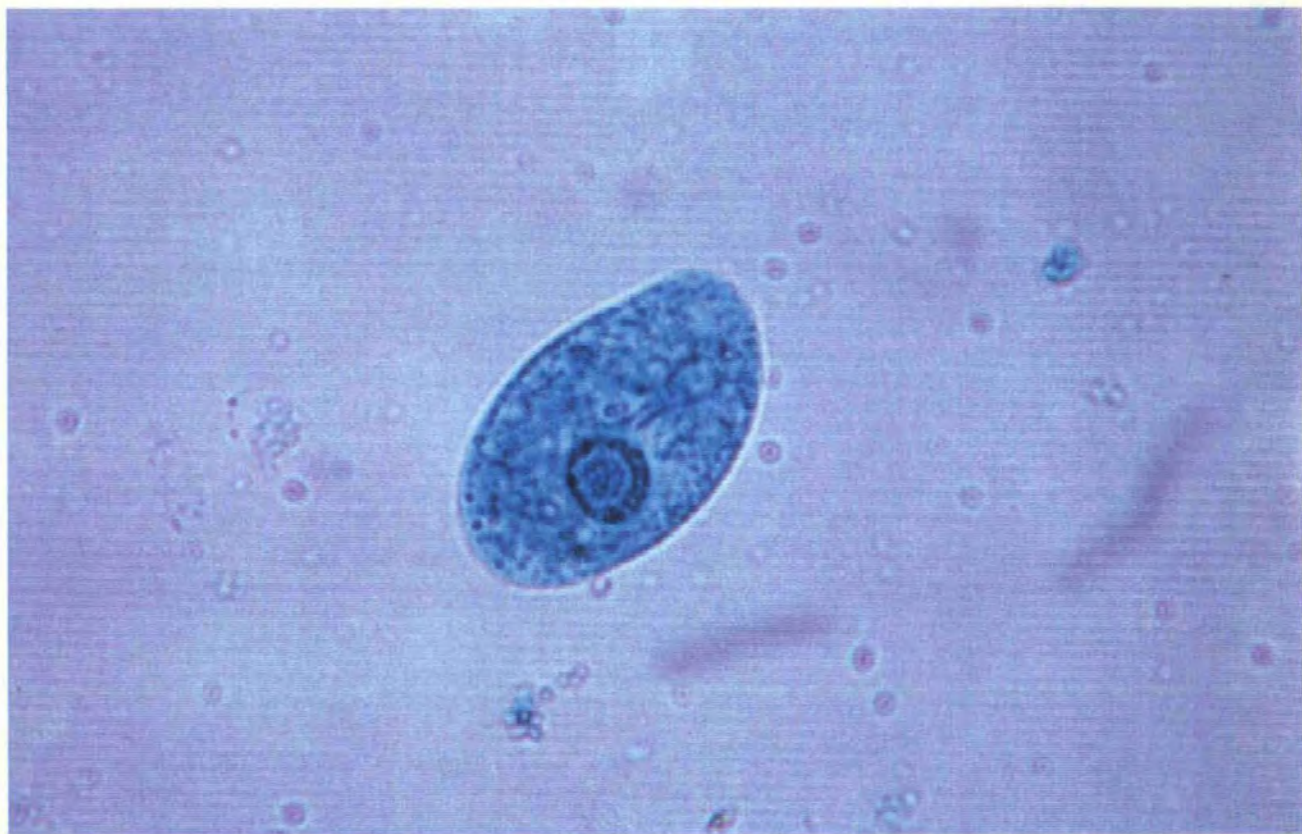


Figure 5 *Oreochromis mossambicus* infected with *Icthyophthirius multifiliis*.



## **Chapter 2**

### **Fish stocks and maintenance.**

A description of each of the species of fish used is provided below, along with a colour plate. The method used to obtain these colour plates is described initially and species descriptions follow. Where the species has been used by other workers in genetic studies a discussion is also included. The general maintenance and culture of these species is then the subject of the third and final part of this chapter.

#### **2.1. Live Fish Photography.**

Live fish were photographed in a 200mm x 50mm x 200mm cm tank which had a dividing piece of glass across its long axis. A thin layer of aquarium gravel covered the base. Fish were caught from their tanks and placed into a small perspex tank (2.4l) containing fresh de-chlorinated water. From here the fish were transferred to another small perspex tank. This was carried out three times to wash the fish of any detritus in the water, since such particles clearly showed up on any photographs taken. Water in the actual photographic tank was siphoned off from a clean, but mature aquarium through a plug of filter wool in the siphon tube to remove any dirt particles. This water was allowed to stand in the photographic tank for at least 24 h to permit any air bubbles on the glass to disperse.

The fish to be photographed was placed between the front glass of the tank and the glass divider. If necessary a small amount of anaesthetic was added to reduce the activity of the fish and facilitate photography. If necessary, the glass partition could be moved forward to reduce the swimming area of the fish. Illumination was provided on one side by means of an angle poise lamp for the purposes of focussing.

A Nikon FM2 camera was used fitted with a micro-Nikkor 105mm lens and a Hoya polarizing filter. Flash lighting was provided via a Vivitar 283 flash gun used off-camera. For some photographs a light sensitive thyristor which fitted onto the camera's hot-shoe was used. This thyristor was set on the closest photographic range. However, in later sessions a Vivitar Varipower module was used in the place of the light sensitive thyristor. This enabled the full power of the flash to be utilized facilitating higher F stops and a greater depth of field. ASA 100 film was used for prints and ASA 50 film for transparencies. When the lighting was controlled by the light sensitive thyristor the shutter speed was 1/125<sup>th</sup> with F stops of 8 and 11 being used without polarizing filter and F2.5 and F4 with polarizing filter. For best results, however, the full power of the flash unit, obtained by using the Varipower unit, enabled a shutter speed of 1/125<sup>th</sup> at F11 with the polarizing filter. The flash was held in a retort stand 20cm above the tank and directed down at an angle of slightly greater than 45°.

## 2.2. Fish stocks.

### 2.2.1. Tropical species.

Several varieties of platyfish were maintained. This species was first described by Günther in 1866 as *Platypoecilus maculatus*. Since then it has also been known as *Poecilia maculata*, but is now well established as *Xiphophorus maculatus*. *X. maculatus* has been widely used in genetic studies since the early 1920's. A wide range of areas has been studied, but especially those of sex determination (e.g. Kallman, 1984), melanomas (e.g. Anders & Anders, 1978) and oncogenes (e.g. Anders *et al*, 1984). One other species of *Xiphophorus* (*Xiphophorus variatus*) was used for experimentation, as well as two species of goodeid.

The first five of the varieties below were obtained as fish imported from commercial fish farms in Singapore. Such fish are bred for their colouration and are produced primarily for the hobbyist aquarist trade. A sixth variety and another species used were obtained from private collectors, the name of whom is given for each species. Each of the platyfish strains bred true with respect to

body colour and, where specified, tail spot pattern. The species and strains were :

1. *X. maculatus* (red platy) - This strain has a red body colouration throughout (Figure 6). King (1975) notes that the red patterning has several components, controlled by different alleles. The fish used here had a red body (*Br*), red dorsal fin (*Dr*), red anal fin (*Ar*) and red caudal fin (*Tr*). Since this was a pure breeding strain it is assumed that the genes at these loci were homozygous. King (1975) also mentions a gene locus '*R*' which controls the red background colour. How King regards this as distinct from the above gene loci is uncertain since he refers to a section on domesticated stocks for further discussion of the '*R*' allele. However, in this section King makes much of the ruby throat (*Rt*) allele, but does not discuss the '*R*' allele. Norton (1967) also refers to the '*R*' allele as causing the red body colour. The red dorsal (*Dr*) allele is also mentioned, but the red body (*Br*), red anal fin (*Ar*) and red caudal fin (*Tr*) are omitted. Despite this confusion it remains that these fish were pure breeding for body colour. On the whole no tail spot patterning was seen in this strain, but occasionally individuals with one spot on the caudal peduncle (*P<sup>O</sup>*) would be seen.

2. *X. maculatus* (red wagtail platy) - Originally derived by introgressive hybridization with *X. helleri* (the swordtail fish) (Gordon, 1946) the extensor genes (*E*) of *X. helleri* interact with the comet tail (*P<sup>Co</sup>*) genes of *X. maculatus* to produce black colouring on all fins (Figure 7). The wagtail pattern is here expressed on a red body colouration (*Br* - see discussion under red platy above). This strain always bred true for body colour and the wagtail pattern. This is the same strain, although a different stock, to that used by Bone (1983) in estimating a heritability value for resistance to *Ichthyophthirius multifiliis*.

3. *X. maculatus* (blue platy) - As the name suggests this fish has a blue/green



body colouration and this strain also possessed the so-called 'Mickey-mouse' tail spot pattern (Figure 8). This comprises of a large central dark spot or moon on the posterior caudal peduncle, with a smaller dark spot above and below. This tail spot pattern can be produced by the homozygous moon complete genotype ( $PMcPMc$ ) or the heterozygote moon and twin spot genotype ( $PMPT$ ). Since this tail spot pattern did not hold true in all the offspring produced, the latter is more likely. Indeed, individuals with only a moon tail or twin spot pattern were produced. The body colouration of blue/green did however breed true, as did the red dorsal colouration ( $Dr$ ). Norton (1967) described this blue iridescence gene as dominant. Since no non-blue/green offspring were produced this gene must be present in the homozygous dominant state.

4. *X. maculatus* (yellow comet tail platy) - This fish has a yellow body colouration that is also sometimes described as lemon or golden (Figure 9). King (1975) lists few alleles for yellow body colouration. Yellow caudal peduncle ( $CPy$ ) is the only one to be seen in these fish. In addition the red dorsal ( $Dr$ ) allele was present.

Black pigmentation along the body of the fish is normally produced by the stipple gene. Gordon (1927) showed that the absence of the stipple melanophores is due to the autosomal recessive gene *st*. Hence *stst* individuals appear golden or yellow. However, Norton (1967) states that this golden colour is dominant to white or 'ghost' body colouration. Here Norton (1967) is referring to the golden variety, described by Gordon (1927,1931). The description there of a clear transparent yellow fish, slightly tinged with pale orange, greenish blue eyes and a dorsal fin flushed with red, is a close resemblance of the strain used here. In 1951 though Gordon stated that he found no experimental evidence for the *g* allele being dominant to white or 'ghost' body colouration. If some evidence had come to light since 1951 Norton

(1967) does not mention it. King (1975) also comments that no cross has been performed to test whether *g* and *st* are alleles or separate gene loci.

The yellow/golden colouration described here is very pale (Figure 9) and as such is likely to be the *stst* genotype rather than the presence of any *CPy* allele for yellow caudal peduncle, which gives a definite deep yellow colouration. Alternatively, it could be the *gg* variety of Gordon (1927,1931). Again, the effect of such genes could be influenced by modifying genes or a gene-environment interaction. However, body colouration did breed true throughout.

The tail spot pattern here is actually present on the extreme upper and lower rays of the caudal fin. Black lines running parallel to the dorsal and ventral edges of the caudal fin taper posteriorly to give the comet appearance, controlled by the *PCo* allele for tail spot patterning. This tail pattern bred true, suggesting a *PCoPCo* population.

5. *X. variatus* (sunset platy) - When young these fish look very much like the *X. maculatus* (red platy) above. On becoming adult though the front half of the body becomes a lighter yellow colour whilst the rear half and caudal fin remains a red colour (Figure 10). Norton (1967) describes these fish as marigold platies. The differences between sunset and marigold platies are given as; the colour of the young fry (grey in sunset platies and yellow in marigold platies) and the presence of faint black stripes or markings (present in sunset platies and absent in marigold platies). The Federation of British Aquarists (F.B.A.S.), however, has recognised descriptions of marigold and sunset platies. Whilst both types possess a red caudal fin, the F.B.A.S. recognises that the dorsal fin is red in sunset platies and yellow in marigold platies. The F.B.A.S. standards, therefore, clearly recognise the fish here as sunset platies. Confusion does exist, though, and since these fish were purchased as sunset platies that is the

name which will be used to describe this strain, bearing in mind the description above and Figure 10. In the majority of these fish no tail spot pattern was exhibited. Some did display the twin spot ( $P^T$ ), one spot ( $P^O$ ) or dot ( $P^D$ ) patterns though.

6. *X. maculatus* (Vera Cruz) - These fish are referred to as the Vera Cruz platy since they originate from an area of eastern Mexico around the Vera Cruz lake. The stocks maintained here were obtained from I. Dibble, who authenticated their origin. The two sexes are dimorphic (Figure 11), the male having a predominately black body, with the black colouration tapering from covering the whole body, from a line between the base of the anal and dorsal fins, to a point at the eye. This pigmentation is controlled by the nigra ( $N$ ) gene, which is common among wild platyfish (King, 1975). The female is more drab in appearance, having a light olive green body, which is similar to the ground colour of the male. Both sexes possess the comet tail markings ( $P^{Co}$ ) and the female has an additional black posterior edge to her anal fin. Females additionally show faint, short, vertical bars on the body, especially when young. These may be due to the stripe sided gene ( $Sr$ ) or the faint expression of the  $N$  allele in the females.

7. *Ameca splendens* (Miller & Fitzsimons, 1971) comes from western Mexico and is a goodeid species. The generic name is derived from the fishes original collection locality - the Rio Ameca. The genus is represented only by this one species, which was originally imported into the U.K. on the 19.10.72 from R.R. Miller (Dawes, 1979), and consisted of six pairs of fish. Only four of these pairs survived, from which a large population was derived and disseminated throughout the U.K. Our fish were obtained in 1983 from John Dawes and have been maintained as a large closed population since then. A large population of these fish is now maintained at this laboratory. Figure 12 shows a young adult

female *A. splendens*. Male fish can be identified by the bright yellow on the outer edge of their caudal fin and a modified anal fin.

8. *Ilyodon xanthusi* is another representative of the Goodeidae, first described by Hubbs and Turner in 1939. In the past *I. xanthusi* has been known as *Characodon furcoidens* and *Balsadichthys xanthusi*. These fish were also obtained from John Dawes, who originally obtained them from Ivan Dibble. *I. xanthusi* is very similar in body shape to *A. splendens*, but is slightly longer and slimmer (Figure 13). The female is similar to the male yet lacks much of the yellow colouring. The anal fin and ventral surface of the male are yellow with the dorsal fin black against the body and yellow above. The caudal fin is yellow speckled with black. The male anal fin is also modified as in *A. splendens*.

9. *Oreochromis mossambicus* was commonly used for maintenance of *I. multifiliis* at 24°C. This mouthbrooding cichlid species is a prolific breeder and therefore, supplies were generally plentiful. These fish were originally obtained from the Institute of Aquaculture at the University of Stirling.

### 2.2.2. Coldwater species

1. Common carp (*Cyprinus carpio*) have been used in genetic studies for many years. The genetics of scale pattern determination was first described in 1928 by Rudzinski (Kirpichnikov, 1981). Many subsequent studies made it possible to identify two pairs of autosomal, unlinked genes, *S* and *N*. Kirpichnikov (1981) and Tave (1986), amongst others, have summarized the present understanding of genetic control of scale pattern in carp. The possible genotypes and phenotypes are shown in Table 1. The *S* allele is dominant over the *s* allele and the dominant *N* allele is lethal in the homozygous state. The lethal effect of the *NN* genotype results in the death of embryos at hatching, or soon after their emergence (Kirpichnikov, 1981).

Fully scaled carp possess a complete covering of small scales (Figure 14). Scattered mirror carp have a reduced number of enlarged scales, which are scattered over the whole body of the fish (Figure 15). Linear mirror carp are very similar to scattered carp, with the exception that the scales show some definite distribution along the lateral line (Figure 16). Leather carp possess very few scales. Those present are the enlarged mirror type and are to be found predominately along the dorsal and ventral surfaces (Figure 17).

The pleiotropic effect of the two pairs of genes has been examined by many workers. Kirpichnikov (1981) summarizes the morphological and physiological characters examined. In addition scattered and leather carp have been compared for their resistance to dropsy, a complicated kidney disorder, with scattered carp appearing more resistant (Merla, 1959; Kirpitschnikov and Faktorovitsch, 1969). The decreased fitness of carp with the *N* allele is common throughout. The *S* gene locus also has a pleiotropic effect, but one which is less pronounced. It has been shown though that in the presence of the *N* allele the action of the *S* allele is generally increased.

Fish used here were obtained from the Hampshire Carp Hatcheries. They were brought in as young fry (4-6 weeks) and were known not to have been exposed to *I. multifiliis*. The fry were produced from two crosses which involved only one female, a scattered mirror carp. Crossing this with a male linear mirror carp yielded scattered mirror, linear mirror and leather carp in approximately equal proportions. This is an interesting ratio since theoretically crossing scattered mirror and linear mirror carp should yield either 50% fully scaled and 50% linear mirror carp, or 25% of each scale type (Table 2). Furthermore, adverse conditions tend to favour the fully scaled phenotype compared to the leather carp (Kirpichnikov, 1945).

The fully scaled fry were obtained by crossing the female scattered mirror carp with a fully scaled male. Since the fry were all fully scaled the genotypes of the parents and offspring are known, because only one cross of a fully scaled and a scattered mirror carp produces 100% fully scaled fry (Table 2). Hence the fully scaled fry must be genotype *Ssnn*, the fully scaled male parent *SSnn* and the scattered mirror female parent *ssnn*. Since this same female crossed with a linear mirror carp produced three of the four scale types then we can also deduce the genotypes of the other fish. Since fully scaled carp were not seen at all in this group, but scattered mirror and leather carp were, it is more likely that the offspring should have yielded 25% of each scale type (Table 2). This would suggest that the male, linear mirror carp parent was genetically *SsNn* and that the offspring were; fully scaled, *Ssnn*; scattered mirror, *ssnn*; linear mirror, *SsNn*; leather, *ssNn*.

The absence of the fully scaled *Ssnn* fry suggests two possibilities. These fish may have died due to some other lethal gene combination in their genotype. Alternatively these fish may be present in the population, but some environmental effect, other gene effect or gene-environment effect has transformed them phenotypically into another scale pattern or patterns. However, the three phenotypes present were approximately equal in number, suggesting that fully scaled carp were never viable. The reason for this deficit in fully scaled carp remains unresolved. This unfortunate situation is principally due to the fact that the few carp hatcheries in the country (compared to trout hatcheries) do not breed for all four scale patterns. Indeed this was the only source that could be found for each scale type from known crosses.

2. Goldfish, *Carrasus auratus*, were used for parasite maintenance at 16°C when insufficient carp (*C. carpio*) were available. These were bought in from wholesalers and retailers and were used primarily because of their low cost compared to other available fish.

## **2.3. Maintenance of fish.**

### **2.3.1. Aquaria.**

Fish were kept in glass aquaria (495mm x 670mm x 235mm), in perspex aquaria (455mm x 255mm x 255mm) or plastic tubs (760mm x 440mm x 330mm). The base was lightly covered with gravel which contained added limestone chips to stabilise the pH. Filtration was performed by polyfoam filters, except for the plastic tubs where Eheim external power filters were used.

### **2.3.2. Plant cover.**

Plant cover was provided in the form of Java moss (*Vesicularia dubyana*). This plant grows well in low light conditions, does not need to be rooted to the basal medium and provides good protection for fry. It could easily be removed during cleaning of aquaria and would grow well under the fluorescent room lighting. This was the only light provided in addition to natural sunlight, which entered the room only on one side.

### **2.3.3. Feeding.**

Fish were fed principally on Tetramin Staple Food diet. However, this was supplemented with live tubifex worms, *Artemia nauplii*, white worms, frozen gamma-irradiated daphnia, frozen gamma-irradiated mysid shrimps, peas and Promin (a high protein pelleted food). This variety was to ensure good condition, good growth rate and to facilitate breeding. The tubifex worms were bought into the laboratory every two weeks and kept under running coldwater. These were repeatedly flushed and not used for at least three days to ensure all detritus was removed.

#### **2.3.4. Water quality.**

To maintain constant and low environmental variance water quality was monitored in all the tanks at regular intervals. Temperature and pH were checked most often. Temperature was kept to 23.5-24.5°C and pH to 7.0-7.8. Adjustments in pH were made by performing water changes. Nitrite, nitrate, ammonia, hardness and copper were also monitored, but less frequently. These measurements were only taken if the fish appeared sluggish or when infections were performed. A Pye Unicam PW 9418 pH electrode was used to measure pH and water hardness was measured with Aquadur test strips. Nitrite, nitrate, ammonia and copper content were each determined with a Hach Camlab test kit.

Every 7-14 days each tank had a 30% water change. This water was siphoned from the gravel to remove detritus and replaced with water which had been standing and aerated for at least 12h, but more commonly for more than 24h, to allow dissolved chlorine to dissipate. Any algae was removed from the tank sides with a brush and the sponges of the polyfoam filters were washed in clean water the following day. Covers of the tanks were periodically cleaned to maintain adequate lighting levels from above.



Figure 6 *Xiphophorus maculatus* (red platy).



Figure 7 *Xiphophorus maculatus* (red wagtail platy).



Figure 8 *Xiphophorus maculatus* (blue platy).



Figure 9 *Xiphophorus maculatus* (yellow comet tail platy).



Figure 10 *Xiphophorus variatus* (sunset platy).



Figure 11 *Xiphophorus maculatus* (Vera Cruz platy).  
Above, male. Below, female.



Figure 12 *Ameca splendens*.



Figure 13 *Ilyodon xanthusi*.





Table 1 Scale pattern genotypes and phenotypes of common carp (*Cyprinus carpio*).

| Genotype.   | Phenotype.             |
|-------------|------------------------|
| <i>SSnn</i> | Fully scaled carp.     |
| <i>Ssnn</i> | Fully scaled carp.     |
| <i>ssnn</i> | Scattered mirror carp. |
| <i>SSNn</i> | Linear mirror carp.    |
| <i>SsNn</i> | Linear mirror carp.    |
| <i>ssNn</i> | Leather carp.          |
| <i>SSNN</i> | Non-viable.            |
| <i>SsNN</i> | Non-viable.            |
| <i>ssNN</i> | Non-viable.            |

Figure 14 Fully scaled carp (*Cyprinus carpio*).



Figure 15 Scattered mirror carp (*Cyprinus carpio*).



Figure 16 Linear mirror carp (*Cyprinus carpio*).



Figure 17 Leather carp (*Cyprinus carpio*).



Table 2 Expected offspring produced from all possible scale pattern crosses of common carp (*Cyprinus carpio*).<sup>1</sup>

| Parental phenotype | Parental genotype | SSnn Scaled | Ssnn Scaled | ssnn Scattered | SSNn Linear | SsNn Linear | ssNn Leather | Un-viable |
|--------------------|-------------------|-------------|-------------|----------------|-------------|-------------|--------------|-----------|
| Scal. x scal.      | SSnn x SSnn       | 100         |             |                |             |             |              |           |
| Scal. x scal.      | Ssnn x Ssnn       | 25          | 50          | 25             |             |             |              |           |
| Scal. x scal.      | SSnn x Ssnn       | 50          | 50          |                |             |             |              |           |
| Scal. x scat.      | SSnn x ssnn       |             | 100         |                |             |             |              |           |
| Scal. x scat.      | Ssnn x ssnn       |             | 50          | 50             |             |             |              |           |
| Scal. x lin.       | SSnn x SSNn       | 50          |             |                | 50          |             |              |           |
| Scal. x lin.       | SSnn x SsNn       | 25          | 25          |                | 25          | 25          |              |           |
| Scal. x lin.       | Ssnn x SSNn       | 25          | 25          |                | 25          | 25          |              |           |
| Scal. x lin.       | Ssnn x SsNn       | 12.5        | 25          | 12.5           | 12.5        | 25          | 12.5         |           |
| Scal. x lth.       | SSnn x ssNn       |             | 50          |                |             | 50          |              |           |
| Scal. x lth.       | Ssnn x ssNn       |             | 25          | 25             |             | 25          | 25           |           |
| Scat. x scat.      | ssnn x ssnn       |             |             | 100            |             |             |              |           |
| Scat. x lin.       | ssnn x SSNn       |             | 50          |                |             | 50          |              |           |
| Scat. x lin.       | ssnn x SsNn       |             | 25          | 25             |             | 25          | 25           |           |
| Scat. x lth.       | ssnn x ssNn       |             |             | 50             |             |             | 50           |           |
| Lin. x lin.        | SSNn x SSNn       | 25          |             |                | 50          |             |              | 25        |
| Lin. x lin.        | SsNn x SsNn       | 6.25        | 12.5        | 6.25           | 12.5        | 25          | 12.5         | 25        |
| Lin. x lin.        | SSNn x SsNn       | 12.5        | 12.5        |                | 25          | 25          |              | 25        |
| Lin. x lth.        | SSNn x ssNn       |             | 25          |                |             | 50          |              | 25        |
| Lin. x lth.        | SsNn x ssNn       |             | 12.5        | 12.5           |             | 25          | 25           | 25        |
| Lth. x lth.        | ssNn x ssNn       |             |             | 25             |             |             | 50           | 25        |

<sup>1</sup>Scal. = Fully scaled carp; Scat. = Scattered mirror carp; Lin. = Linear mirror carp; Lth. = Leather carp.

## Chapter 3

### Preliminary studies.

#### 3.1. Introduction and aims.

In attempting to observe the genetics of susceptibility to *Ichthyophthirius multifiliis* the extent of several components needs to be determined. Genetic variation may exist both in the fish stocks under investigation and in the parasite population. The variation can also be confounded by other factors. The various species described in chapter 2 come in differing shapes and sizes. Even on one fish there are different surfaces upon which the parasite may rest : the fins and the body surface. The level of exposure to the parasite must be standardised, since variation in exposure will result in variations in infection levels which are not genetically related. As in any study of this nature, it is also a requirement that infections performed at different times can be compared. The aim of these initial control studies is, therefore, to answer the following questions;

1. How can infection levels on different size/shaped fish be compared ?
2. What is the best exposure level to ensure that a suitable number of parasites can be counted accurately ?
3. Do infections repeated over a period of time give similar results ?
4. Is there any evidence for the presence of tank effects ?

## 3.2. *Ichthyophthirius multifiliis*.

### 3.2.1. Parasite maintenance.

*I. multifiliis* was obtained on recently imported tropical fish species, or from a fish farm in South Devon. Infected fish were placed in a 455mm x 255mm x 255mm perspex aquarium, filtered by a polyfoam filter and with a thin layer of aquarium gravel on the base. Tropical strains of the parasite were kept at  $24.0 \pm 1.0^\circ\text{C}$  and temperate strains were kept at  $16.0 \pm 1.0^\circ\text{C}$ . All aquaria were shielded from direct sunlight. Between 2 and 10 infected fish were placed in a recently assembled aquarium, with an equal number of previously uninfected fish. At  $24^\circ\text{C}$  this was mainly comprised of *Oreochromis mossambicus*, although other surplus fish were occasionally used, notably *Xiphophorus maculatus*. At  $16^\circ\text{C}$  common carp (*Cyprinus carpio*) and common goldfish (*Cyprinus auratus*) were used to passage the parasite.

Infections were generally allowed to occur naturally in the passage tank, unless the parasite density on the fish was low. In the latter case fish, infected with mature parasites, were placed in a 180mm x 115mm x 115mm aquaria to concentrate the theronts produced. The parasites were allowed to leave the host and then the fish was removed. When microscopic examination proved that theronts were being released, previously unexposed fish were then added to the 180mm x 115mm x 115mm tank for upto 5h. After this time all the contents of the 180mm x 115mm x 115mm tank were emptied back into the main passage tank.

Temperature in the passage tank was maintained at  $24.0 \pm 1.0^\circ\text{C}$  and continually monitored, by way of a digital thermometer attached to the outside of the aquarium. Water changes were performed every two weeks, but only when the parasite was known to be developing upon the host. One possible problem with infected fish is the development of secondary infections, but these were very rarely observed and only ever consisted of opportunistic fungal pathogens. Four 455mm x 255mm x 255mm aquaria were set aside from all other aquaria for passage of the tropical parasite isolates, with two other such tanks being



used for the temperate parasite isolates. Infected fish were rotated among these tanks so that each passage aquaria could be completely cleaned at approximately 3 month intervals.

### 3.2.2. Parasite collection.

*I. multifiliis* was collected for controlled infections by placing several heavily infected fish into a 500ml beaker, containing 300ml of filtered aquarium water, for 25-30 minutes. Mature parasites were quite freely dislodged from the host by body movements of the fish whilst in close proximity. This process could be aided by the addition of a little aeration to the beaker to cause water turbulence. Parasites were never collected by mechanical means, such as body scrapes. The mature trophozoites thus obtained were incubated at  $24.0 \pm 1.0^\circ\text{C}$ , in the case of the tropical parasite strains, or  $16.0 \pm 1.0^\circ\text{C}$ , in the case of the temperate parasite strains.

### 3.2.3. Determination of parasite concentration.

A Sedgewick-Rafter counting cell (S-R cell) was used to evaluate theront concentrations. The S-R cell has a volume of 1ml, divided into 1,000 squares, arranged in 50 columns of 20 squares, each square having a volume of  $1 \mu\text{l}$ . To determine the accuracy of theront concentration estimation, using the S-R cell, repeated counts were made from a single batch of incubated parasite. Two replicate counts on each sample were made. Each count consisted of the sum of five columns of the S-R cell, or 100 squares (*i.e.* 0.1 ml). This procedure was repeated on fifteen 1ml samples. Therefore, in total, theronts in 3ml of water, taken from a total sample of 15ml of water, were counted.

A total of 438 theronts were counted, suggesting an overall theront concentration of 146 theronts per ml for the 3ml in which theronts were counted. An analysis of variance was performed to,

a. Determine whether the two replicate counts for each sample differed significantly, and

b. To determine whether the fifteen 1ml samples differed significantly.

In both cases  $F$ -tests were not significant at the 10% level (a.  $F=1.164_{[14;59]}$ , b.  $F=0.864_{[14;45]}$ ). Hence, the two replicate counts were not significantly different and the fifteen 1ml samples were not significantly different. In determining theront concentrations, then, the sum of five columns of the S-R cell (*i.e.* 0.1ml) is sufficient. Such a sampling procedure will not be significantly different to sampling 3ml.

#### 3.2.4. Infection protocol.

Fish, which were to be infected with *I. multifiliis*, were allowed at least 24h to acclimatise to the 455mm x 255mm x 255mm infection tank. Total water changes, using dechlorinated water, had been carried out in these aquaria 24h prior to the introduction of fish. A thin covering of gravel, a polyfoam filter and a small amount of plant cover (Java moss, *Vesicularia dubyana*) were provided in each aquarium. *I. multifiliis* was collected and theront concentrations determined as above. Fish were infected in opaque 500ml beakers with a known number and concentration of theronts for 30 minutes. During this time infection occurs (McCallum, 1982). The contents of the beaker were then all returned to the aquarium in which the fish had been acclimatised. Water quality parameters were determined at this point, as they also were upon termination of the experiment, when the parasite reached maturity upon the host.

#### 3.2.5. Infection level determination.

Since Bone (1983) had previously shown distributions of trophozoites on the left and right side of host fish to be not significantly different, only the left side of the fish was chosen for assessing infection levels. The time at which such levels were determined depended upon water temperature, but was the point at which the parasite had almost reached maturity on the host. At 24°C this was at 3 days after infection, but at 19°C it was at 6 days and at 16°C 10 days after infection. Fish were lightly anaesthetised with benzocaine (ethyl *p*-aminobenzoate). To ensure accurate counting the body was divided into the

areas shown in Figure 18. Evaluation of infection levels was performed under a binocular microscope (magnification x7 - x45). Initial investigations established the accuracy of such a procedure by repeated counts on several fishes. Body area was measured using a method adapted from Webb (1970). Body circumference was measured at 0.5 cm intervals by using lengths of cotton thread. From these measurements a flat area could be derived, equivalent to that of the body area of the fish. This area was evaluated using a program, written in BASIC, on a BBC microcomputer (Appendix A). In addition to this, dorsal, caudal and anal fins were laid flat on paper and drawn around with a marker pen. These marked areas were then evaluated using a Calcomp digitizer linked to a Prime computer. This data was transferred to the BBC microcomputer via the file transfer program Kermit and was then analysed alongside the body area data above (see Appendix A).

### 3.3. Data analysis.

Data analysis provides some problems when large numbers of factors are involved, e.g. time of experiment, temperature of experiment, surface area of the fish. A complete data set for such an experiment would be orthogonal, i.e. the inclusion of a term *A* in the analysis will have the same reduction in residual variance, whether or not term *B* is already in the analysis (the reverse also being true). Hence, the sum of squares would be independent of the order of analysis in the anova table :

$$\begin{aligned} \sum (\text{Sum of squares due to temperature of the experiment} + \\ \dots + \text{Sum of squares due to surface area}) = \\ \sum (\text{Sum of squares due to surface area} + \\ \dots + \text{Sum of squares due to temperature of the experiment}). \end{aligned}$$

Data sets are not always strictly orthogonal, with the result that analysis of the effects in a single analysis of variance table would differ, depending upon the order in which those effects were entered into the anova table. Non-

orthogonality is due to at least one correlation between two, or more, of the factors involved in the experimental procedure. For example, such correlations could be due to the correlation between a tank and its temperature. Hence, variation due to temperature is due to variation between tanks, resulting in non-orthogonality between these two variables.

Upon advice from a statistician, it was decided that the best, conservative approach, to this problem was a series of anova tables to determine the sums of squares of each effect in turn, given all other effects. For example, the sums of squares for variation due to surface area differences could be determined given effects due to time of the experiment, temperature of the experiment and sex of the fish. In this way the *minimum* variation attributable to each factor would be determined, any correlated variation already being explained in the variation due to all other factors. One consequence of such a method of analysis is that correlation among factors gives rise to an effect known as aliasing (Baker & Nelder, 1978). For example, if several tanks of fish are compared, where each tank is at a different temperature, then the factor of tank also explains variation due to the factor of temperature.

The analysis was performed upon a Prime computer using version 3.77 (1985) of the Generalised Linear Interactive Modelling (GLIM) package produced by the Royal Statistical Society. Where applicable, pairwise comparisons were performed using a *t*-test of difference between two levels of the same parameter. Like all *t*-tests, this was computed from the division of the difference in the means of estimated values by the standard error of that difference.

### **3.4. *Ameca splendens*: variation in infection level with dose.**

#### **3.4.1. Experimental procedure.**

To determine an appropriate parasite exposure level a series of experiments were performed, exposing *A. splendens* to four different concentrations of *I. multifiliis* : 2,000 theronts per fish (40 theronts per ml), 3,000 theronts per fish (60 theronts per ml), 6,000 theronts per fish (120 theronts per ml) and 12,000

theronts per fish (240 theronts per ml). Infections were performed as in section 3.2.4 above. Only a single isolate (isolate A, Appendix B) was used in this investigation. Between 17 and 28 fish were infected at each concentration.

It is possible that the size of the surface area over which infection could occur may result in a significant amount of variation in infection levels, e.g. smaller fish may have relatively more parasites than larger fish, or vice versa. Therefore, the surface area of 35 specimens of *A. splendens* was determined (see section 3.2.5). Linear regressions of total and standard length on surface area were performed. Where surface area was not directly measured, it was then estimated, as the mean of the values computed from standard and total lengths.

### 3.4.2. Results.

Nine tanks of *A. splendens* were infected : 2 at 2,000 theronts per fish (20 fish), 2 at 3,000 theronts per fish (20 fish), 3 at 6,000 theronts per fish (28 fish) and 2 at 12,000 theronts per fish (17 fish). Where surface area was directly measured it averaged  $3.73 \pm 0.97 \text{ cm}^2$ . Standard lengths measured  $3.2 \pm 0.4 \text{ cm}$  and total lengths measured  $3.5 \pm 0.4 \text{ cm}$ . Linear regressions of standard and total length with surface area were both highly significant ( $P < 0.001$ ).

Relationships were ;

$$\text{Surface area (cm}^2\text{)} = 2.13 \text{ S.L. (cm)} - 3.35$$

$$\text{Surface area (cm}^2\text{)} = 1.95 \text{ T.L. (cm)} - 3.51$$

Computations of surface area values from length measurements produced an overall average surface area of  $3.43 \pm 0.88 \text{ cm}^2$ .

Infections were carried out at five different points in time over a 2 month period. Temperatures during these infections ranged from 22.7 to 25.2°C (mean =  $24.0 \pm 1.0^\circ\text{C}$ ). Infection levels, for each tank and dose, are given in Table 3. Infection levels varied from 88 trophozoites to 712 trophozoites on a single fish. Mean infection levels rose from 158.4 trophozoites per fish, at 2,000 theronts per fish, to 457.0 trophozoites per fish, at 6,000 theronts per fish. Figure 19

shows the increased infection levels with parasite dose. At high infection levels, of over approximately 400 trophozoites per fish, evaluation of the number of parasites became more difficult, as the trophozoites clumped together in 'grape-like' fashion. Hence, at high infection levels, of upto 12,000 theronts per fish, mean infection levels apparently fell to 349.0 trophozoites per fish.

Analysis of the infection levels was performed using the GLIM package on a Prime computer (Table 4). Variation due to temperature was not significant ( $P > 0.10$ ) over the recorded range (22.7 - 25.2°C). The variation due to the time of the experiment, surface area of the fish and dosage of the parasite were, however, all significant at the 0.1% level. The five points in time covered a period of 62 days and, therefore, temporal variation in infection levels was seen over a 2 month period. Infections occurred at intervals of 7, 21, 10 and 24 days. The relationship between surface area and infection level was one of a factor of  $0.17 \pm 0.03$ , implying that a unit increase in surface area is only accompanied by a 17% increase in infection level. Hence, larger fish have relatively less parasites than smaller fish, when area is taken into account.

The significant variation due to dose of the parasite is the major point of interest in this investigation. This variation had a much larger mean square value (5.557) than the other three variables (area mean square = 1.078, time mean square = 0.449, temperature mean square = 0.009). All pairwise comparisons of infection levels by dose were aliased,<sup>2</sup> except for the 2,000 vs 3,000 theronts per fish comparisons. This was highly significant ( $t = 12.803$ ,  $P < 0.001$ ). Therefore, increasing parasite exposure concentrations, upto 6,000 theronts per fish, produced a significant increase in infection levels above which accurate counting becomes impossible due to the clustering of trophozoites.

<sup>2</sup>See section 3.3 for a discussion on aliasing

### **3.5. *Ameca splendens* : variation in infection levels between tanks.**

#### **3.5.1. Introduction.**

In comparing different populations of fish for their susceptibility to *I. multifiliis* one is presented with two possible modes of experimentation. In the first the populations are all maintained within the same environment (*i.e.* tank) and in the second the populations are maintained in similar, but separate, environments (*i.e.* tanks). These two approaches both possess advantages and disadvantages, which are fundamental to all conclusions derived from such experiments. In the first instance the populations of fish are subjected to identical environmental conditions and, therefore, there can be no between population environmental variation. Nevertheless, under such conditions social interactions between populations are to be expected. Even a physical barrier, such as netting, between populations of fish cannot remove such interactions with certainty. The occurrence of visual, hormonal and even electrical social interaction between fish is well known. The alternative of keeping each population in separate, yet similar, environments has but one disadvantage. This is commonly known as the tank effect and is the variation attributable to the uniqueness of each particular aquarium. The extent of this variation may or may not be significant but must be evaluated if the maintenance of each population in separate aquaria is to be considered.

#### **3.5.2. Experimental procedure.**

To determine the extent of any tank effects four identical aquaria were set up, each containing 10 *A. splendens*. Infections, using a dose of 2,000 theronts per fish, were performed as above, using only a single batch of parasite which was all derived from a single parasite isolate (isolate G, Appendix B). On the third day the experiment was terminated, with infection levels and surface areas of each fish being determined. An analysis was then performed using the GLIM package on a Prime computer (see section 3.3).

### 3.5.3. Results.

The mean surface area of fish in each of the four aquaria was very similar - 2.71, 2.88, 2.71 and 2.61 cm<sup>2</sup>. Temperatures were constant throughout the experimental period ( $25.2 \pm 0.2^\circ\text{C}$ ). The balance of the sexes was heavily skewed towards females, with a male to female ratio of 1 : 5. Males harboured  $256.5 \pm 71.0$  trophozoites per fish, whilst female fish harboured  $259.7 \pm 65.6$  trophozoites per fish. Parasite burdens varied overall from 115 to 441 trophozoites per fish. The tank mean infection levels were  $268.1 \pm 72.1$ ,  $205.6 \pm 70.6$ ,  $286.9 \pm 45.2$  and  $275.8 \pm 45.8$  trophozoites per fish.

The series of analysis of variance tables produced by the GLIM package are to be found in Table 5. The effects of surface area, sex of the fish and tank effect were all included in the analysis. Temperature was not included, since it was constant, with a maximum range, among all the four tanks, of only  $0.5^\circ\text{C}$ .

The variation due to the sex of the fish was not significant ( $P > 0.10$ ), therefore negating the possibility of any effect due to the high imbalance of the male : female ratio. The variation due to the surface area of the fish was the strongest effect ( $F_{[1,34]} = 43.765$ ,  $P < 0.001$ ) upon infection level. Hence, the relatively large range of 2.82 cm<sup>2</sup> in surface area provided a significant source of variation in parasite burden.

The tank effect was the main point of interest here. This effect was significant at the 0.1% level ( $F_{[3,34]} = 13.397$ ). Closer examination of this variation, using pairwise comparisons, clearly shows that only one tank was responsible for this significant effect. The tank with a mean infection level of  $205.6 \pm 70.6$  trophozoites per fish was significantly different ( $P < 0.001$ ) from the other three tanks (mean =  $276.9 \pm 54.4$  trophozoites per fish), which formed a homogeneous group ( $P > 0.10$ ). Thus, although tank effects are perhaps minimal, there is always the possibility that, in occasional tanks, the tank environment may influence infection level despite attempts to minimise such environmental variance.



### 3.6. Discussion.

This investigation clearly shows that the observed variation in *I. multifiliis* infection levels is composed of four components : variation due to time, variation due to surface area, variation due to tank effects and variation due to exposure level. Variation in exposure levels of *I. multifiliis* contained the major part (mean squares = 5.557, Table 4), of the total explained variance. Four infection levels of 2,000, 3,000, 6,000 and 12,000 theronts per fish were used. Infection levels rose from  $158.4 \pm 55.6$  trophozoites per fish at 2,000 theronts per fish, through  $266.6 \pm 62.7$  trophozoites per fish at 3,000 theronts per fish, to  $457.0 \pm 126.4$  trophozoites per fish at 6,000 theronts per fish. The reason for the drop in infection levels at 12,000 theronts per fish to  $349.0 \pm 71.9$  trophozoites per fish is, undoubtedly, due to the clustering of parasites. At such high exposure levels the large numbers of parasites gather together in 'grape-like' bunches. Hence, infection levels could not be quantified with the an acceptable degree of accuracy. The lower infection level of the 12,000 theronts per fish infections is, therefore, quite probably due to the underestimation of infection levels by the omission of hidden trophozoites. Observations suggested that the accuracy of quantification was hindered in such a way when parasite levels exceeded 400 trophozoites per fish. Indeed the level of infection at 12,000 theronts per fish (a mean of 349 trophozoites per fish) is perhaps a better estimation. Maximum infection levels recorded at each dose level were : 2,000 theronts per fish - 256 trophozoites per fish, 3,000 theronts per fish - 396 trophozoites per fish, 6,000 theronts per fish - 712 trophozoites per fish and 12,000 theronts per fish - 514 trophozoites per fish. Based on the criteria that infection levels obtained should fall below 350 trophozoites per fish, a standardised infection level of 2,000 theronts per fish is suggested for future investigations.

The significant amounts of variation due to surface area, in the two infection studies here, occurred over total ranges of  $4.41 \text{ cm}^2$  and  $2.82 \text{ cm}^2$ . Since the effect of surface area was significant within one species here, the requirement that it should be included in further analyses is essential. If different species and sizes of fish are to be compared, for their susceptibility to *I. multifiliis*

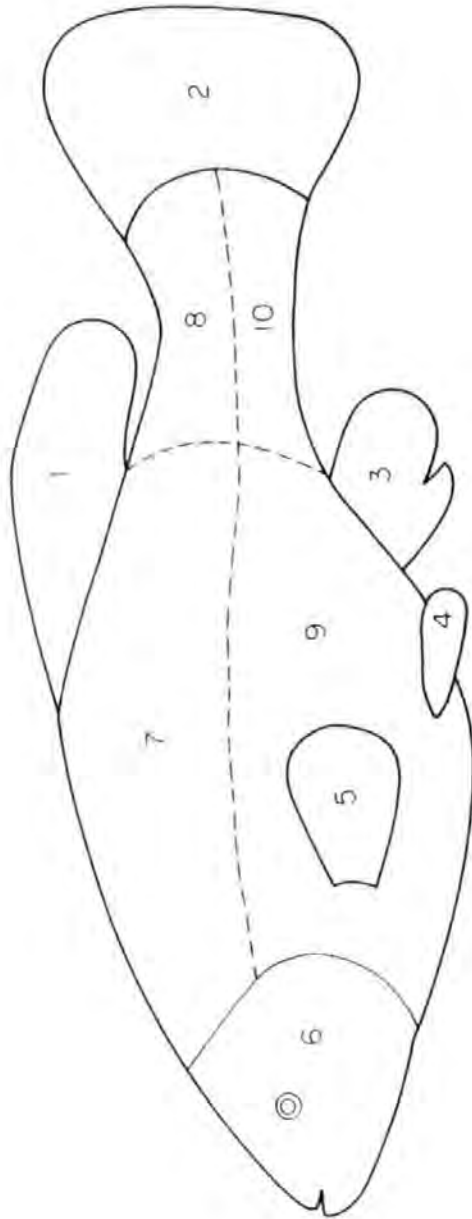
infection, it is vital that incorrect conclusions are not drawn due to the use of different sized and shaped species.

The variation due to the time of the experiment is significant ( $P < 0.001$ ), although why this is so is unclear. The experiments occurred over a period of 2 months at 7, 21, 10 and 24 days intervals. Temporal variation was significant even at the infections 7 days apart ( $P < 0.001$ ). Infections first occurred in early May and were completed by early July. Although an explanation for such variation is not clear, it is obvious that one needs to be aware of this variation and incorporate it into an analysis of susceptibility to *I. multifiliis*. Variation due to temperature was not significant ( $P > 0.10$ ) in the parasite dose study and the variation in temperature was low ( $24.0 \pm 1.0^\circ\text{C}$ , mean squares = 0.009). Temperature will continue to be monitored in future experiments, however, to ensure stable environmental conditions remain.

Although significant variation was found for tank effects, this was all attributable to one tank out of the four examined. The mean infection level for this tank was  $205.6 \pm 70.6$  trophozoites per fish, compared to values of  $268.1 \pm 72.1$ ,  $286.9 \pm 45.2$  and  $275.8 \pm 45.8$  trophozoites per fish for the other three tanks. Tank effects, therefore, whilst not widespread, were present. This is another important factor to consider when discussing infection levels of *I. multifiliis*. The experimental method of separate tanks for each group of fish is still the preferred method, though, since alternative approaches are more likely to provide other, more complex, problems.

Having determined an appropriate exposure level and the significance of other factors involved in the infection process, it is now possible to observe the variation in susceptibility to *I. multifiliis* due to genetically controlled factors. This will be pursued in several ways throughout the rest of this study.

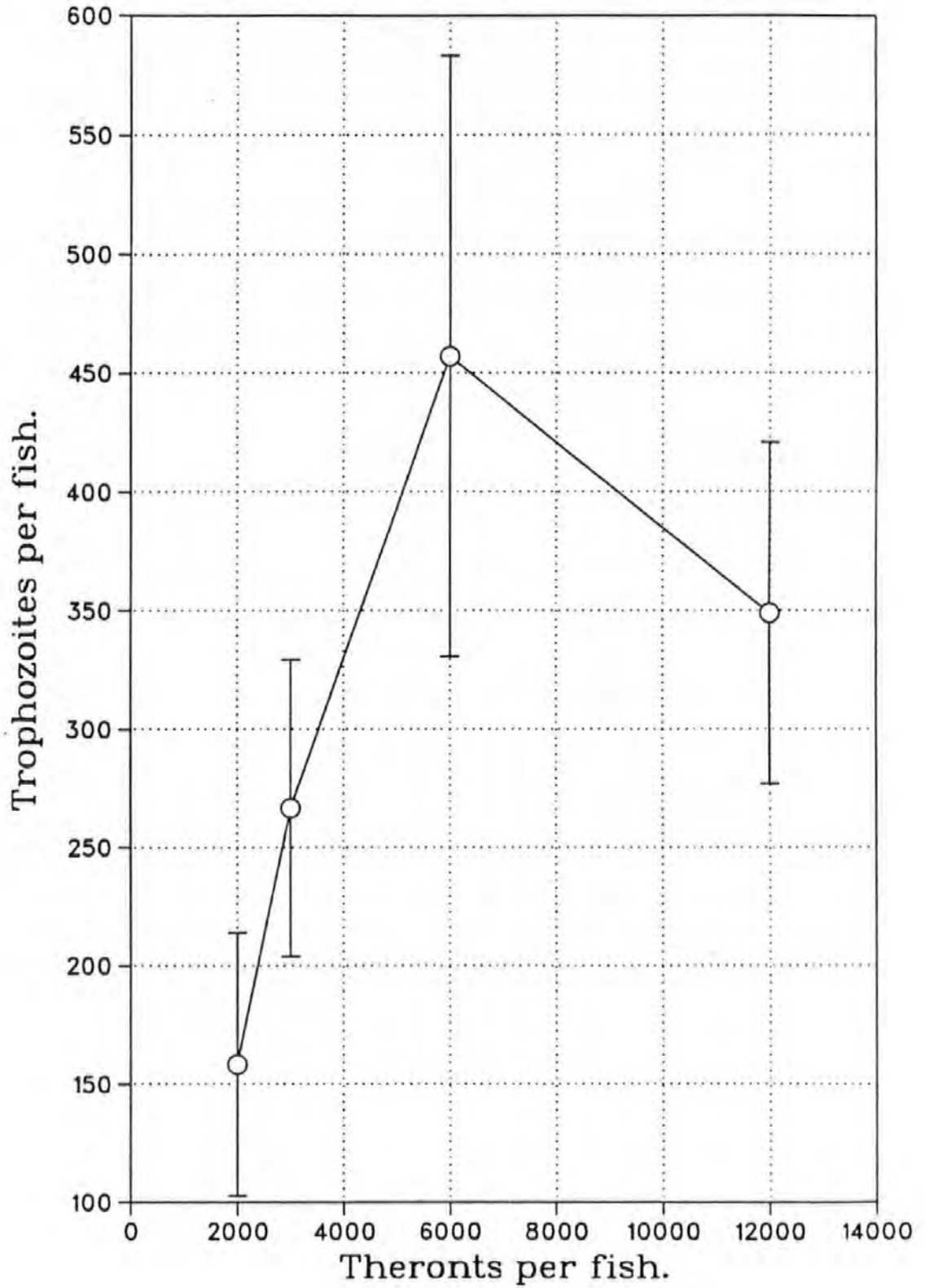
**Figure 18** Division of body surface area into ten different sections to aid assessment of infection levels.



**Table 3** Parasite burdens on *Ameca splendens* exposed to four concentrations of *Icthyophthirius multifiliis*.

|                       | Date.   | Infection level (Trophozoites per fish). |               |
|-----------------------|---------|--|---------------|
|                       |         | Tank.                                    | Dose.         |
| 2,000 theronts/fish.  | 11/6/85 | 196.7 ± 51.4                             | 158.4 ± 55.6  |
|                       | 5/7/85  | 120.1 ± 25.1                             |               |
| 3,000 theronts/fish.  | 1/6/85  | 250.1 ± 65.6                             | 266.6 ± 62.7  |
|                       | 1/6/85  | 283.2 ± 58.3                             |               |
| 6,000 theronts/fish.  | 11/5/85 | 557.1 ± 106.7                            | 457.0 ± 126.4 |
|                       | 11/5/85 | 457.7 ± 91.0                             |               |
|                       | 5/7/85  | 345.2 ± 79.0                             |               |
| 12,000 theronts/fish. | 4/5/85  | 357.2 ± 91.3                             | 349.0 ± 71.9  |
|                       | 4/5/85  | 341.7 ± 54.1                             |               |

Figure 19 Increasing *Ichthyophthirius multifiliis* burdens on *Ameiops splendens* with increasing exposure levels.



**Table 4** Analysis of variance tables for each of the variables in infections of *Ameba splendens* with four doses of *Icthyophthirius multifiliis*.<sup>2</sup>

| Source of variation.                       | d.f. | Sum of Squares. | Mean Squares. | F      | P    |
|--|------|-----------------|---------------|--------|------|
| Effects due to area, temperature and time. | 6    | 12.091          | 2.015         | 59.41  | **** |
| Effects due to dose.                       | 1    | 5.557           | 5.557         | 163.82 | **** |
| Residual.                                  | 77   | 2.612           | 0.034         |        |      |
| Total.                                     | 84   | 20.260          |               |        |      |
| Effects due to dose, temperature and time. | 6    | 16.570          | 2.762         | 81.41  | **** |
| Effects due to area.                       | 1    | 1.078           | 1.078         | 31.78  | **** |
| Residual.                                  | 77   | 2.612           | 0.034         |        |      |
| Total.                                     | 84   | 20.260          |               |        |      |
| Effects due to dose, area and time.        | 6    | 17.639          | 2.940         | 87.79  | **** |
| Effects due to temperature.                | 1    | 0.009           | 0.009         | 0.269  | NS   |
| Residual.                                  | 77   | 2.612           | 0.034         |        |      |
| Total.                                     | 84   | 20.260          |               |        |      |
| Effects due to dose, area and temperature. | 5    | 16.750          | 3.350         | 98.76  | **** |
| Effects due to time.                       | 2    | 0.898           | 0.449         | 13.24  | **** |
| Residual.                                  | 77   | 2.612           | 0.034         |        |      |
| Total.                                     | 84   | 20.260          |               |        |      |

The level of  $P < 0.10$  is included to highlight results which are bordering on being significant at the 5% level and may benefit from increased sample size in future work.

<sup>2</sup>Significance levels : P < 0.10 - \* ; P < 0.05 - \*\* ; P < 0.01 - \*\*\* ; P < 0.001 - \*\*\*\*.

**Table 5** Analysis of variance tables for each of the variables in four identical infections of *Ameba splendens* with *Icthyophthirius multifiliis* to determine the extent of tank effects.<sup>3</sup>

| Source of variation.          | d.f. | Sum of Squares. | Mean Squares. | F      | P    |
|-------------------------------|------|-----------------|---------------|--------|------|
| Effects due to area and sex.  | 2    | 1.009           | 0.504         | 17.177 | **** |
| Effects due to tank.          | 3    | 1.180           | 0.393         | 13.397 | **** |
| Residual.                     | 34   | 0.999           | 0.029         |        |      |
| Total.                        | 39   | 3.188           |               |        |      |
| Effects due to area and tank. | 4    | 2.151           | 0.538         | 18.309 | **** |
| Effects due to sex.           | 1    | 0.038           | 0.038         | 1.307  | NS   |
| Residual.                     | 34   | 0.999           | 0.029         |        |      |
| Total.                        | 39   | 3.188           |               |        |      |
| Effects due to sex and tank.  | 4    | 0.904           | 0.226         | 7.695  | **** |
| Effects due to area.          | 1    | 1.285           | 1.285         | 43.765 | **** |
| Residual.                     | 34   | 0.999           | 0.029         |        |      |
| Total.                        | 39   | 3.188           |               |        |      |

<sup>3</sup>Significance levels as in Table 4.

## Chapter 4

### Inter- and intra- specific variation to ichthyophthiriasis.

#### 4.1. Introduction and aims.

To evaluate the level of any variation in resistance to parasitic infection several species and strains were exposed to *Ichthyophthirius multifiliis*. The infection procedure was kept constant throughout and environmental variance was kept to a minimum. Hence, it would be expected that any inter- or intra- specific differences would be due to genetic variation.

This genetic variation, however, may be composed of variation on the part of the fish host and variation due to the use of several isolates of parasite. There appears to be a limited period of time over which an isolate can be kept through serial passage in the laboratory. Being a ciliate, freeze storage of recognised strains of the parasite is not possible at the present time. In addition, the parasite population dies out after a period of time, despite the addition of previously unexposed hosts. This has been found by other workers (Houghton, 1987; Matthews & Matthews pers. comm.; and Wahli & Schmitt, pers. comm.) as well as in this study. This problem means that any long-term experiment necessitates the use of more than one isolate of parasite.



## 4.2. Methods.

### 4.2.1. Experimental protocol.

Infections were carried out, as detailed in chapter 3, at a dose of 2000 theronts per fish and concentration of 40 theronts per ml. A tank of ten *A. splendens* was always infected to facilitate comparisons. Water quality was measured prior to and upon termination of the experiment, on the third day.

It was attempted to use as few a number of parasite isolates as was practical. However, strains of parasite only last a finite period of time in the laboratory and from time to time a fresh stock of parasite had to be obtained. Several management rules were followed on this point. Batches of parasite were never maintained together in the laboratory. For these experiments batches of *I. multifiliis* were always obtained from recently imported tropical species of fish. Maintenance periods for each batch of parasite were recorded and hence the batch code for each infection is known. Appendix B lists the maintenance periods and codes for each batch.

### 4.2.2. Within species between strains comparisons.

Several strains of *Xiphophorus maculatus* were compared for their resistance to infection. These strains, which are described in more detail in chapter 2, were the yellow comet tail platy, the red platy, the red wagtail platy and the blue platy. All of these strains were domesticated strains, imported from Singapore and obtained from local retail outlets.

Fish were only obtained which were known not to have been infected with *I. multifiliis* whilst in Britain. The fish were regularly checked at the importers for a period of two weeks after importation. If no disease outbreak had occurred during this time the fish were then used in the laboratory. A further precaution was taken in that these fish were not used for infection but were first bred. The offspring produced provided both greater numbers of fish and fish which were known to have never been in contact with *I. multifiliis* from birth. As a

consequence of this, the extent of any possible maternal effects, as reported by Bone (1983) and Price & Bone (1985), will have been greatly reduced.

#### 4.2.3. Between species comparisons.

Each of the strains of *X. maculatus* above were compared with three other species. The most closely related species was *Xiphophorus variatus* (the sunset platy). *Ilyodon xanthusi*, a goodeid species, closely related to *Ameioba splendens*, was also included. Infection procedures were the same as those in the section above.

#### 4.2.4. Method of analysis.

The analysis was performed upon a Prime computer using the Generalised Linear Interactive Modelling (GLIM) package (see chapter 3, section 3.3). Factors included were of time of the experiment, temperature of experiment, surface area of the fish, sex of the fish, isolate of the parasite and strain/species of fish. The data set here is not strictly orthogonal, with the result that analysis of the effects in a single analysis of variance table would differ, depending upon the order in which those effects were entered into the anova table. The reason for this non-orthogonality is that there is at least one correlation between two, or more, of the factors involved in the experimental procedure. Such correlations are likely to be due to the use of different parasite isolates at different times, the use of different strains / species of fish at different times, and, hence, the use of different parasite isolates on different strains / species of fish. These correlations of time, parasite isolate and strain / species of fish used are unavoidable practical problems.

### 4.3. Results.

Water quality parameters showed minimal variation (temperature =  $24.1 \pm 1.0^\circ\text{C}$ , pH =  $7.4 \pm 0.2$ ). The surface area values for the strains and species of fish used varied considerably, from 1.54 cm<sup>2</sup> to 11.10 cm<sup>2</sup> (overall mean =  $4.94 \pm 2.05$  cm<sup>2</sup>). *A. splendens* had the lowest mean surface area of

3.36±1.0 cm<sup>2</sup>, with *X. maculatus* (yellow comet tail platy) having the largest surface area of 7.36±0.80 cm<sup>2</sup>. The distributions of the sexes among all strains / species of fish was of the ratio 1:1.8 for males : females.

Infection levels were seen to fluctuate with the time of year at which the experiment was performed (Figure 20). However, the relative infection levels of strains and species remained constant at different times of infection, with the exception of *A. splendens* relative to *X. maculatus* (red platy). These two species were infected simultaneously on three occasions, the third occasion involving a different parasite isolate (isolate H) to the previous two occasions (isolate G). *A. splendens* harboured less parasites than *X. maculatus* (red platy), except for the infection using parasite isolate H. Infections were of a similar magnitude for both infections with parasite isolate G : *A. splendens*, time 1 - 38.0±14.4 trophozoites per fish, time 2 - 21.3±6.7 trophozoites per fish ; *X. maculatus* (red platy), time 1 - 48.0±4.1 trophozoites per fish, time 2 - 55.4±13.1 trophozoites per fish. On the third occasion of infection, with parasite isolate H, two tanks of *X. maculatus* (red platy) and a single tank of *A. splendens* were infected. Infection levels were, *A. splendens* 267.8±131.0 trophozoites per fish, *X. maculatus* (red platy) 112.7±47.0 and 135.1±12.2 trophozoites per fish. Hence, infection levels were higher and, with this parasite isolate, *A. splendens* now harboured more parasites than *X. maculatus* (red platy).

Infections were first attempted for this study in mid-September, but either produced no mature parasites on exposed hosts or insufficient theronts were produced to even attempt infections, until the first half of December. Two isolates of parasite (isolates C and D, Appendix B) were kept over this period, but neither produced any successful experimental infections. These isolates were kept for 52 and 14 days respectively. The third isolate used, isolate E, was kept for 36 days and produced 2 successful and 2 unsuccessful infections on *A. splendens* and *I. xanthusi*. This parasite isolate was, however, lost on the last day of December. Parasite isolate F was obtained the same day from recently imported fish, but this isolate was never kept for more than a single passage. Isolate G, however, obtained on the 13<sup>th</sup> January, survived in the

laboratory for 151 days. In this time the vast majority (65%) of the infections reported below were performed. Infection levels were below 120 trophozoites for all strains and species, until mid-April, after which infection levels rose as high as 441 trophozoites. A subsequent single unsuccessful infection was followed by the loss of this isolate in mid-June. The isolate of parasite that followed was kept for 160 days, but during most of this time was producing only very small numbers of theronts. On two occasions it was considered that this parasite isolate had been lost, only for it to reappear several days later. Two unsuccessful and two successful infections were achieved, in late July and late September. When successful, infection levels were in the range of 37 to 588 trophozoites per fish (mean= $130.9 \pm 93.0$  trophozoites per fish). This parasite isolate was then lost by the 26<sup>th</sup> November and no further infections were performed for this investigation. Overall *A. splendens* was infected with parasite isolates E, G and H, whilst *I. xanthusi* was infected with isolates E and H and *X. maculatus* (red platy) and *X. variatus* (sunset platy) were infected with isolates G and H. All other strains and species of fish were only ever infected with a single parasite isolate.

Taking mean infection levels for species and strains, irrespective of any of the above temporal effects and not taking into account the different sizes of surface area, then differences in infection levels can be seen. The highest infection levels were found on *A. splendens*, with a mean of 134.7 trophozoites per fish. This group also has the largest standard deviation, of 111.9 trophozoites per fish, and coefficient of variation, 88.3%. Two platyfish species had the lowest counts :  $38.9 \pm 25.4$  trophozoites per fish for *X. maculatus* (blue platy) and  $39.6 \pm 18.3$  trophozoites per fish for *X. variatus* (sunset platy). The coefficient of variation for *X. variatus* (sunset platy) was 46.3% and was the lowest such coefficient recorded. The four strains of *X. maculatus* had coefficients of variation from 50.2 to 65.3%. *I. xanthusi* had a higher coefficient of variation of 77.5%. There are, therefore, higher coefficients of variation in stocks which have been directly derived from the wild, i.e. *A. splendens* and *I. xanthusi*. The ordering of the mean total parasite count data from least to most resistant is as follows: *A. splendens*, *X. maculatus* (red platy), *I. xanthusi*, *X. maculatus* (red

wagtail platy), *X. maculatus* (yellow comet tail platy), *X. variatus* (sunset platy) and *X. maculatus* (blue platy). To determine the significance of the differences between the strains / species of fish and to include effects due to the size of the fish and time of the experiment, however, requires an analysis of variance to be performed.

#### 4.3.1. Analysis of the results.

One of the fundamental assumptions of analysis of variance is that the variance is independent of the mean. One indication of this is to plot the residual values (*i.e.* the difference between actual and estimated values) against the estimated values, where a random distribution should be obtained if the variance is independent of the mean. Figure 21 shows that for untransformed data a non-random distribution was obtained via this method. A square root transformation produced a better distribution, but a natural logarithmic transformation produced the most random distribution. Therefore, in all the analysis below logarithmic data was used.

Analysis of major effects was not possible in the case of variation due to different isolates, since only a single isolate was ever used at any particular time. Hence the isolate covariate is also explained by the time covariate. Such dependence among covariates gives rise to the effect known as aliasing (Baker & Nelder, 1978). The data here are said to be extrinsically aliased since the sample data does not contain sufficient information to estimate all parameters. Variation due to sex of the fish was not significant ( $P > 0.10$ ) (Table 6). All other effects were significant *i.e.* variation due to time of the experiment, ( $P < 0.001$ ), variation due to temperature of the experiment ( $P < 0.05$ ), variation due to surface area of the fish, ( $P < 0.001$ ) and variation due to the strain/species of fish, ( $P < 0.001$ ).

The variation with time occurred over a period of 294 days. Maximum infection levels, which were usually on *A. splendens*, varied from a low of 67 trophozoites per fish, in late February and mid-March, to a high of 558 trophozoites per fish, in late September. The large variation in this effect

therefore stresses the importance of incorporating the time of the experiment into the analysis.

The significance of temperature effects is surprising, given the small amount of variation in temperature ( $24.1 \pm 1.0^\circ\text{C}$ ). The analysis used individual tank temperatures and, therefore, temperature and tank effects are synonymous. This will be discussed below in more detail.

The significance of the variation in surface area values stresses the importance of incorporating a measure of fish size in the analysis of infection levels. The slope of the relationship between surface area and parasite burden, given all other factors, was estimated by the GLIM package as  $0.14 \pm 0.02$ . This slope is significantly different from zero ( $P < 0.001$ ). Surface areas varied considerably ( $1.54$  to  $11.10 \text{ cm}^2$ ) and, therefore, one can only be certain of differences in resistance, of different sized species of fish, when area is taken into account. Even within species a 100% increase in surface area is coupled to a 14% increase in infection levels. Hence, the inclusion of surface area is important, even when only a single species is involved.

Given the significant variation due to different strains / species of fish pairwise comparisons using  $t$  values were performed, (Table 7). As a consequence, the various strains/species used here can be divided into three distinct groups (Figure 23). The first, most susceptible group, consists solely of *A. splendens*, where infection levels were recorded as  $134.7 \pm 111.9$  trophozoites per fish. The differences between *A. splendens* and the other strains/species were always significant at the 5% level. The second group was comprised of *I. xanthusi*, *X. maculatus* (red platy), *X. maculatus* (yellow comet tail platy) and *X. maculatus* (red wagtail platy). Here infection levels were recorded as  $71.2 \pm 55.2$ ,  $89.0 \pm 44.7$ ,  $50.2 \pm 28.3$  and  $67.0 \pm 37.5$  trophozoites per fish respectively. Again differences between this and the other two groups were significant at the 5% level. The third and final group is comprised of *X. maculatus* (blue platy) and *X. variatus* (sunset platy). This group was highly significantly different from all others, ( $P < 0.001$ ), with recorded infection levels of  $38.9 \pm 25.4$  and  $39.6 \pm 18.3$

trophozoites per fish respectively. Significant intra- and inter-specific variation in resistance to *I. multifiliis* infections was therefore observed.

It is also possible to analyse interaction terms between covariates. Analysis of major effects determines whether infection levels differ at the different levels of that factor. Interaction analysis, however, determines whether changes in another factor affects all the levels of the major factor in a similar manner. Three interaction terms were analysed; species-temperature interactions, species-isolate interactions and isolate-temperature interactions. Figure 22 shows that these, non-orthogonal interaction terms, had only a minimal effect upon the relationship of variances and means, which remained random.

The analysis of species-temperature effects was significant at the 0.1% level. The GLIM package estimated the slopes of the regression line, for parasite burden and temperature, for each species. For *A. splendens*, *I. xanthusi*, *X. maculatus* (red wagtail platy) and *X. variatus* (sunset platy) these were not significantly different from zero ( $P > 0.10$ ). The slopes of the lines for the red and blue *X. maculatus* strains were significantly different from zero at the 0.1% level, whilst that for the yellow comet tail *X. maculatus* was significant at the 5% level. Slopes for the yellow comet tail and red *X. maculatus* strains were positive ( $0.41 \pm 0.19$  and  $0.64 \pm 0.09$  respectively), but not significantly different from each other ( $P > 0.10$ ). The slope of the line for the blue *X. maculatus* strain, however was negative ( $-1.42 \pm 0.34$ ) and highly significantly different from the regressions for yellow comet tail and red *X. maculatus* ( $P < 0.001$ ). This is interesting since temperatures were relatively constant ( $24.1 \pm 1.0^\circ\text{C}$ ) throughout the course of the experiment. Hence, the strains / species of fish used here respond, in different ways, to even small temperature changes. The division of the strains / species of fish into three distinct groups does not appear to have any pattern, e.g. strain or species groups. Some doubt must, therefore, be expressed about the significance of this interaction analysis.

Species-isolate interactions were also significant at the 0.1% level, although insufficient information from the infection protocol prevented many

comparisons. It will be recalled that only *A. splendens*, *I. xanthusi*, *X. maculatus* (red platy) and *X. variatus* (sunset platy) were infected with more than a single parasite isolate (page 68). The only possible comparison which proved to be significant was that for *X. maculatus* (red platy) and *A. splendens*  $P < 0.001$ . Species-isolate effects for the other species were not significant ( $P > 0.10$ ). This effect was noticeable when the experiments were being performed. In the majority of cases *A. splendens* was the species which carried the highest parasite burdens. The one exception to this was the infections with *A. splendens* and *X. maculatus* (red platy). These two species were infected, at the same time, on three occasions. Apart from time, the only difference was the isolate of parasite used, both species of fish being derived from identical stocks at each time. On one of these occasions the *X. maculatus* (red platy) had a higher parasite burden than *A. splendens*. A species-isolate interaction between *X. maculatus* (red platy) and parasite isolates G and H was, therefore, suspected, and was confirmed in the above analysis.

Isolate-temperature effects were found to be not significant at the 10% level.

#### 4.4. Discussion.

In any study on disease resistance there are many factors to consider. Indeed a measure of the effectiveness of such studies is how successfully the effect of these factors can be isolated and quantified. The aim of this study was to evaluate several strains and species of fish for their resistance to *I. multifiliis*. An experimental approach was adopted whereby the effects of several environmental variables could be evaluated. Hence, fish were subjected to identical conditions in terms of water source, filtration, position within the laboratory etc.

Any host-parasite relationship is, however, far more complex than this. A hypothesis was proposed that one or more of the following may affect the infection level of *I. multifiliis* on its' host : size of the fish, sex of the fish, strain/species of fish and isolate of parasite . Taking into account these effects, the role of genetic variation of the host and genetic variation of the parasite can



be discussed. The extent of these and environmental factors are discussed, in turn, below.

#### Time of the experiment.

Variation due to the time of year the experiment was performed was highly significant ( $P < 0.001$ ). Indeed this effect stood out when the experiments were being conducted. Reasons for this effect are unclear, although a plot of infection levels against time shows an interesting trend (Figure 20). For all species, no infections were successful in the early part of the year until mid-March. Infection levels obtained were then constant until early June, when infection levels rose by about 300%. Following this, infections were not successful until mid-August, and then only for a short period. Little can be said about September and early October, since no infections were attempted, but infections were successfully performed by late October. Successful infections had also been performed in December of the previous year, with infection levels of August, October and December being intermediate between the peak of early June and the plateau of mid-March to late May.

What is most interesting about this trend is that it follows that found in the wild (Wagner, 1960 ; Meyer, 1974). The usual reasoning behind this annual cycle is that low temperatures in winter preclude any outbreaks of ichthyophthiriasis. When temperatures rise in March/April, however, outbreaks occur reaching a peak in early summer (Butcher, 1941 ; Wagner, 1960 ; Meyer, 1974 ; Lahav & Sarig, 1973). Throughout the main part of the summer months *I. multifiliis* is not often recorded despite the higher temperatures, since it is now argued that the fish are in a much improved state of health compared to their late over-wintering state. In addition, the majority of fish are now immune to infection following earlier infections. Reasons for the later resurgence of outbreaks in the autumn are unclear, but the phenomenon has been recorded (Butcher, 1941 ; van Duijn, 1973 ; Lahav & Sarig, 1973).

Here temperatures were relatively constant ( $24.1 \pm 1.0^\circ\text{C}$ ) and certainly did not vary annually to the same extent that they do in ponds/rivers. It is suggested,

therefore, that temperature is not the major factor controlling this annual cycle of infections, as once thought. Quite what might control this annual cycle is unclear and further discussion will be held over until Chapter 8.

#### Temperature of experiment.

It has already been stated that temperatures were constant throughout and, therefore, it is perhaps surprising that, with a temperature range of 21.9 - 25.5°C, effects were significant. However, given that temperatures differed between tanks, but not within tanks, then temperature is also a measure of tank effects. Rather than each tank being coded by a number, each tank is effectively coded by its' temperature. The reasons for tank effects are numerous and varied. Other water quality parameters, besides temperature, were also stable, suggesting something more subtle, such as ambient lighting levels, is the cause.

One way to negate tank effects is to place more than one species in each tank. Ideally all seven strains/species of fish would have been infected within the same tank. Variation due to tank effects would then have been zero, but in such a situation species interactions are extremely likely. Individuals of *A. splendens*, especially the males, have been known to harass other, smaller species. The establishment of such a dominance hierarchy in a mixed species tank could well influence infection levels, through increased stress in submissive fish and through damaged tissue during hierarchical disputes. The approach used here was, therefore, considered the optimum approach, with the benefit that tank effects could be quantified and accounted for in strain/species comparisons.

#### Size of fish.

Often the comparisons made between different species of fish are done on the basis of length. This does not take into account though the fact that fish may differ in body shape. For example, here *A. splendens* and *I. xanthusi* are slender fish, whilst the xiphophorid fish used are more rounded in body shape.

Therefore, differences in size are more realistically compared when in the form of surface area on which the parasite may attach.

The hypothesis that larger fish harbour more parasites was found to be true. A doubling of surface area though does not imply a doubling of parasite burden, but an increase of  $14 \pm 2\%$ . Hence, smaller fish are *relatively* more susceptible. It is, therefore, not valid to compare infection levels of *I. multifiliis* on different species, or to compare juvenile and adult fish, unless size, preferably in terms of surface area, is taken into consideration. No previous work by other authors has been found, where infection levels were recorded over more than a very small fraction of the body and where surface area was also recorded. Therefore, the comparison of previous reports where *I. multifiliis* levels were determined, but surface area was not, is quite subjective.

#### Sex of fish.

Differences due to the sex of the fish were not significant. This suggests that any genetic control of resistance is not linked to genes which also control sex determination. Pickering & Christie (1980) examined brown trout, during the period of the spawning season. Sexually mature male fish were found to be more heavily infected with *I. multifiliis*, *Scyphida*, *Gyrodactylus* and *Saprolegnia* than either mature female or immature fish. Pickering & Christie suggest several reasons for this sexual difference, including the reduced production of mucous by male fish during spawning. The situation with brown trout differs markedly from the situation with the species used here, in that brown trout have a clear reproductive period with associated genetically controlled physiological changes. The species used here, however, breed throughout the year and show no obvious similar physiological changes.

#### Isolate of parasite.

It was not possible to compare the four tropical strains of parasite used here, whilst also taking into account variation due to all other factors. This is undoubtedly due to the fact that no two isolates were used at any one time.

Hence, the parasite isolate data is correlated with the time of the experiment data. In this experiment isolates were only replaced when the previous isolate had died out, to prevent possible cross-contamination of isolates. Therefore, the data <sup>are</sup> termed extrinsically aliased, since the variation due to the time of the experiment also explains variation due to different isolates of the parasite. It is not possible then to comment upon variation due to different tropical isolates of the parasite.

Analysis of isolate-temperature interaction terms, however, was possible, but was not significant ( $P > 0.10$ ). This suggests that the isolates used here perform in a similar manner over the somewhat narrow temperature range encountered (3.6°C). Nevertheless, the effect of such a narrow temperature range in itself was significant, as discussed above.

#### Strains/species of fish.

The principal aim of this study was to assess the extent of inter- and intra-specific variation in resistance to ichthyophthiriasis. The division of the strains/species used here into three significantly different groups clearly demonstrated the presence of inter- and intra-specific differences. Between species differences were recorded between each of the four species used (*i.e.* *A. splendens*, *I. xanthusi*, *X. maculatus* and *X. variatus*). Within species differences were less widespread, with three of the four strains of *X. maculatus* differing from the fourth.

One might surmise that *A. splendens* was the most susceptible species since it is one of the two wild types used, as opposed to the domesticated strains of *X. maculatus*. In support of this, the most resistant group was comprised purely of the domesticated species *X. maculatus* (blue platy) and *X. variatus* (sunset platy). Outbreaks of ichthyophthiriasis are more common in the confined environs of captivity than in the wild. Hence, intentionally or unintentionally, some degree of selection for resistance is exercised in the domestication process (Doyle, 1983). Ståhl (1983) examined 37 enzyme loci on 1,643 tissue samples from natural and hatchery populations of atlantic salmon. Samples

from hatchery stocks had a significantly lower amount of genetic variability than natural populations, indicating a loss of genetic variation through domestication. Gall (1983), in summarizing a symposium open discussion on fish genetics, commented that many had argued that hatchery propagation had resulted in a loss of genetic variation. Although not a direct measure of genetic variation, it is interesting to note that coefficients of variation were also lowest for the domesticated platies. Selection for resistance to *I. multifiliis* is, therefore, likely to have occurred on the fish farms in Singapore, from where these domesticated platy fish strains were imported.

The two species-interaction terms examined here were also significant. The species-temperature interactions, when analysed in detail, provided two significantly different groups. The slopes of the relationship between temperature and infection level for the yellow comet tail, red and blue strains of *X. maculatus* were  $0.41 \pm 0.19$ ,  $0.64 \pm 0.09$  and  $-1.42 \pm 0.34$  respectively. These slopes were all significantly different from zero ( $P < 0.05$ ), but those for the remaining species were not ( $P > 0.10$ ). The two slopes for the yellow comet tail and red *X. maculatus* strains were not significantly different from each other ( $P > 0.10$ ), but were significantly different from the blue *X. maculatus* strain ( $P < 0.001$ ). It is thought unlikely that such a small variation in temperature could be responsible for such an effect.

Moav (1976) recognised five major groups of genotype-environment interactions : 1. genotype-pond interactions, 2. genotype-season interactions, 3. genotype-age interactions, 4. genotype-husbandry system interactions, and 5. genotype-competition interactions. Examples of these are cited by Moav (1976) and further reports of genotype-environment interactions have been produced since this time (see, for example, Wohlfarth *et al*, 1983). A species-environment interaction does appear to be present here and this must be borne in mind when attempting genetical improvement of stock in a variety of environmental situations.

The significant species-isolate interactions for *X. maculatus* (red platy)

confirmed expectations, since some variation in infection levels in this stock had been noticed prior to analysis of the data. Such variations may well reflect variation in the parasite. The significance of this effect highlights some of the difficulties in transferring the results obtained here to the general aquaculture situation. Such problems will be discussed further in the final discussion chapter. The absence of species-isolate interactions in other species is partly due to the use of only one isolate of parasite with a species precluding such species-isolate interaction comparisons.

The approach used here provides no detail of the type of difference between strains/species of fish, except to say that it is present and is considered to be due to genetical effects. The nature of this genetic variation is now the subject of the following chapters.

**Figure 20** Variation in infection levels of *Ichthyophthirius multifiliis* on exposed fish during the period late 1985 to the end of 1986. All fish were exposed to the parasite in a similar manner and at the same dosage level. The use of different parasite isolates is marked.

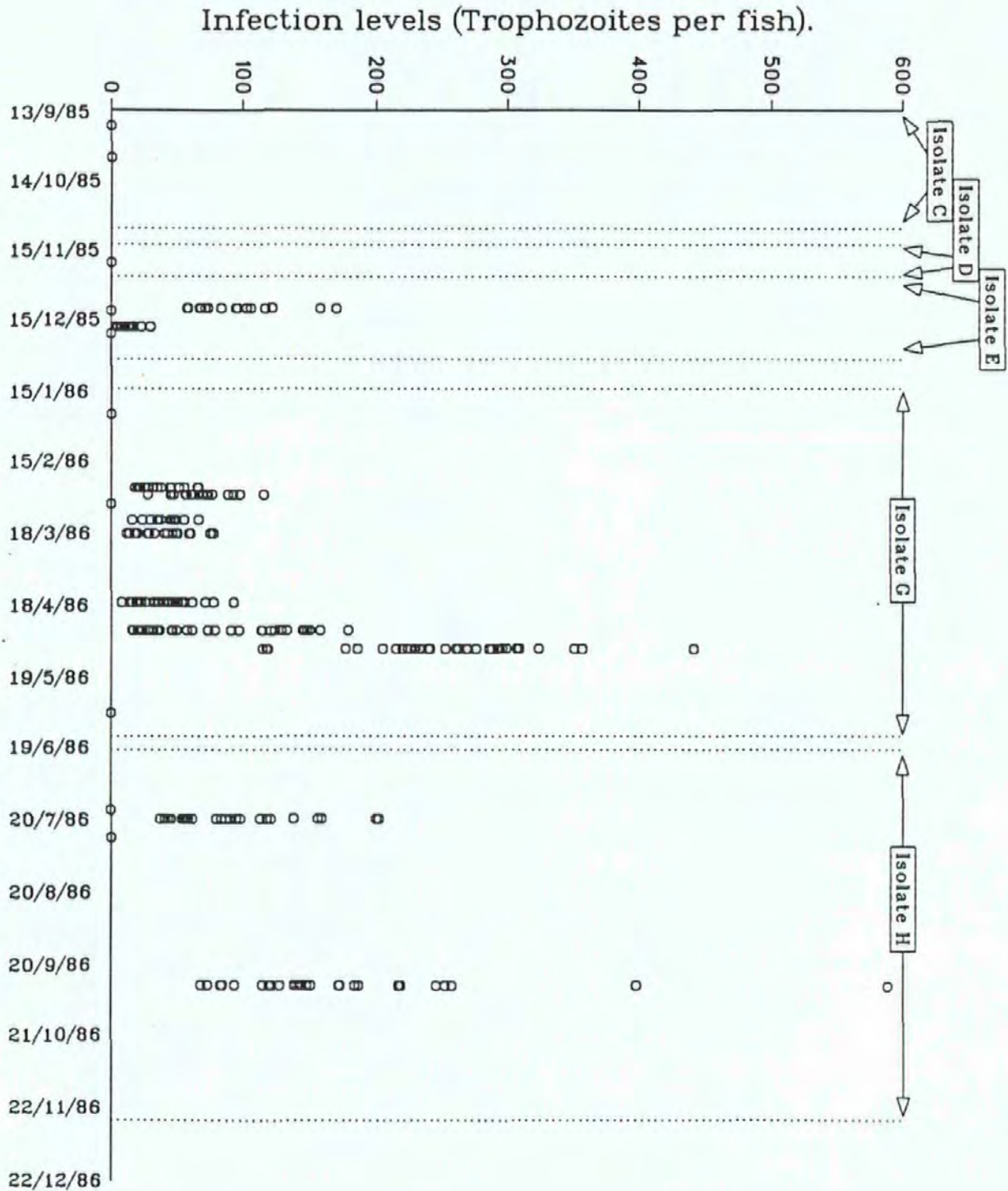


Figure 21 Plots of residual values against fitted values after analysis for total *Ichthyophthirius multifiliis* burden on *Xiphophorus maculatus* (Strains : red platy, red wagtail platy, yellow comet tail platy and blue platy), *Xiphophorus variatus* (sunset platy), *Ilyodon xanthusi* and *Ameca splendens*.  
a. Untransformed data, b. Square root transformed data, c. Natural logarithmic transformed data.

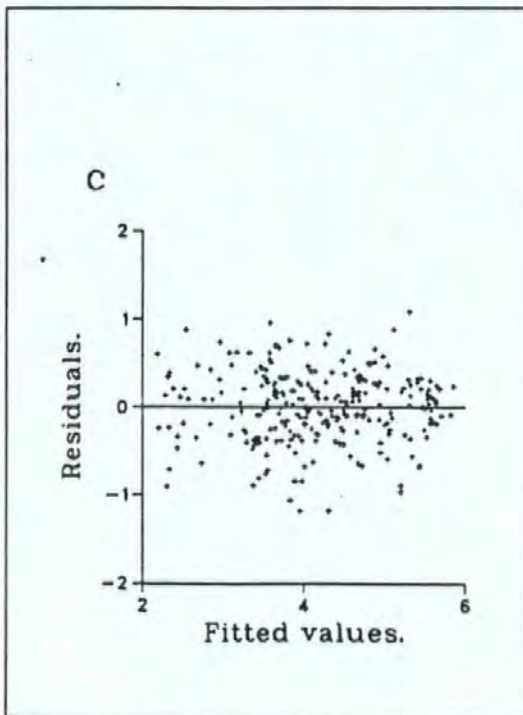
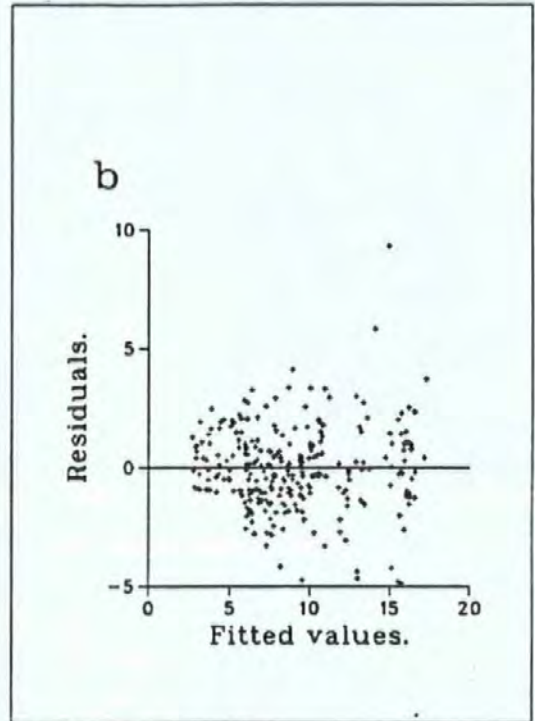
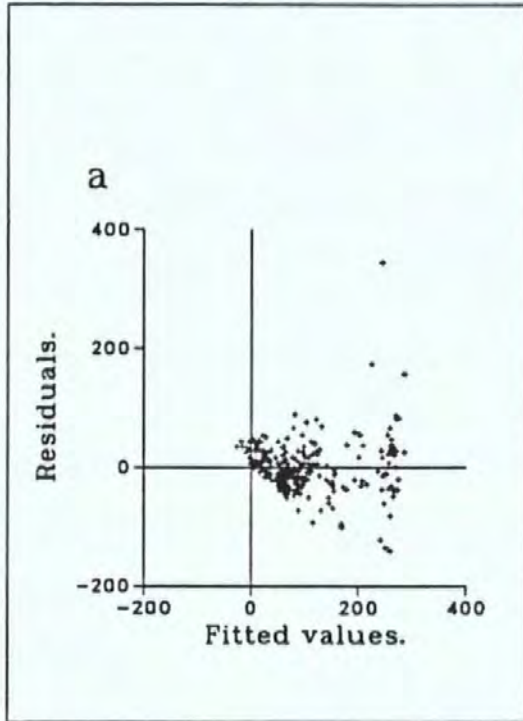
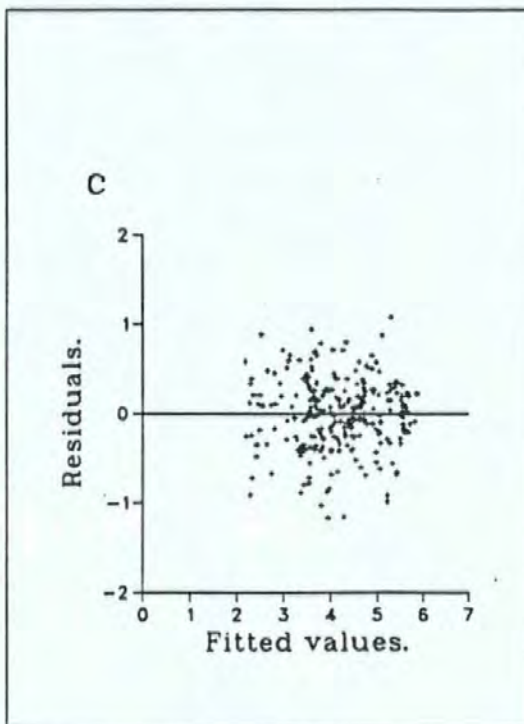
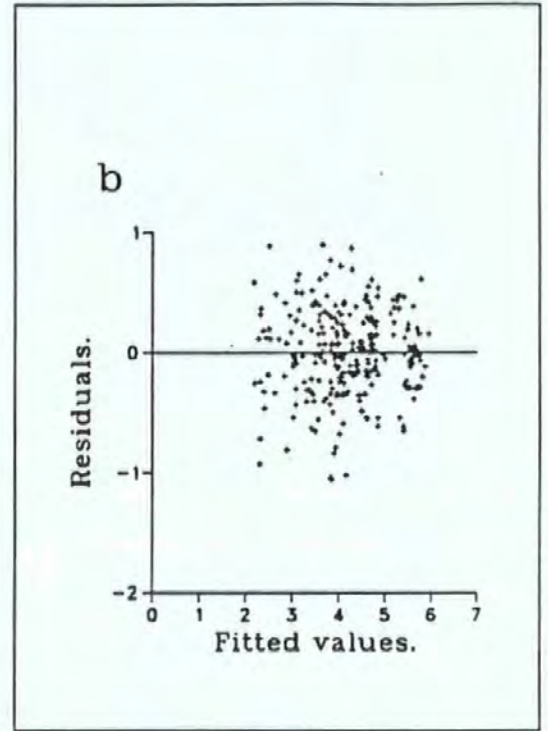
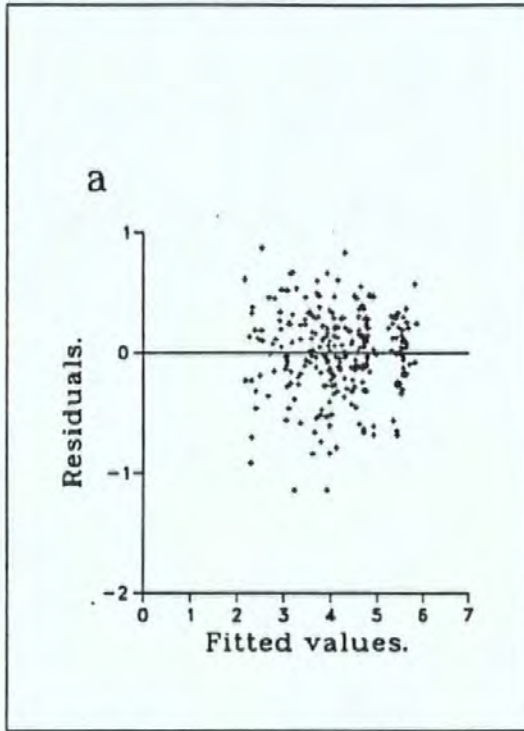




Figure 22 Plots of residual values against fitted values after interaction term analysis using natural logarithmic transformed data for total *Ichthyophthirius multifiliis* burden on *Xiphophorus maculatus* (Strains : red platy, red wagtail platy, yellow comet tail platy and blue platy), *Xiphophorus variatus* (sunset platy), *Ilyodon xanthusi* and *Ameca splendens*. a. Species-temperature interactions, b. Species-isolate interactions, c. Isolate-temperature interactions.



**Table 6** Analysis of variance tables for each of the variables in infections of *Xiphophorus maculatus* (red platy, red wagtail platy, yellow comet tail platy and blue platy), *Xiphophorus variatus* (sunset platy), *Ameca splendens* and *Ilyodon xanthusi* with *Ichthyophthirius multifiliis*.<sup>4</sup>

| Source of variation.   | d.f. | Sum of Squares. | Mean Squares. | F    | P    |
|--|------|-----------------|---------------|------|------|
| Effects due to sex, area, strains/species, isolate and temperature.  | 11   | 88.9            | 8.1           | 49.6 | **** |
| Effects due to time.   | 8    | 121.9           | 15.2          | 93.6 | **** |
| Residual.  | 255  | 41.5            | 0.2           |      |      |
| Total.   | 274  | 252.3           |               |      |      |
| Effects due to time, area, strains/species, isolate and temperature. | 18   | 210.8           | 11.7          | 71.9 | **** |
| Effects due to sex.  | 1    | 0.03            | 0.03          | 0.2  | NS   |
| Residual.  | 255  | 41.5            | 0.2           |      |      |
| Total.   | 274  | 252.3           |               |      |      |
| Effects due to time, sex, strains/species, isolate and temperature.  | 18   | 204.6           | 11.4          | 69.9 | **** |
| Effects due to area.   | 1    | 6.2             | 6.2           | 38.1 | **** |
| Residual.  | 255  | 41.5            | 0.2           |      |      |
| Total.   | 274  | 252.3           |               |      |      |
| Effects due to time, sex, area, isolate and temperature.             | 13   | 192.7           | 14.8          | 91.1 | **** |
| Effects due to strains/species                                       | 6    | 18.0            | 3.0           | 18.5 | **** |
| Strain/species - isolate interactions.                               | 3    | 8.3             | 2.8           | 17.0 | **** |
| Strain/species - temperature interactions.                           | 6    | 11.5            | 1.9           | 11.8 | **** |
| Residual.  | 255  | 41.5            | 0.2           |      |      |
| Total.   | 274  | 252.3           |               |      |      |

<sup>4</sup>Significance levels as in Table 4.

Table 6 continued ...

| Source of variation.   | <i>d.f.</i> | Sum of Squares. | Mean Squares.          | <i>F</i> | <i>P</i> |
|--|-------------|-----------------|------------------------|----------|----------|
| Effects due to time, sex, area, strains/species and temperature. | 19          | 210.8           | 11.1                   | 67.6     | ****     |
| Effects due to isolate.  | 1           |                 | Extrinsically aliased. |          |          |
| Isolate - temperature interactions.                              | 2           | 0.2             | 0.1                    | 0.5      | NS       |
| Residual.  | 255         | 41.5            | 0.2                    |          |          |
| Total.   | 274         | 252.3           |                        |          |          |
| Effects due to time, sex, area, strains/species and isolate.     | 18          | 209.9           | 11.7                   | 71.6     | ****     |
| Effects due to temperature.                                      | 1           | 0.9             | 0.9                    | 5.3      | ***      |
| Residual.  | 255         | 41.5            | 0.2                    |          |          |
| Total.   | 274         | 252.3           |                        |          |          |

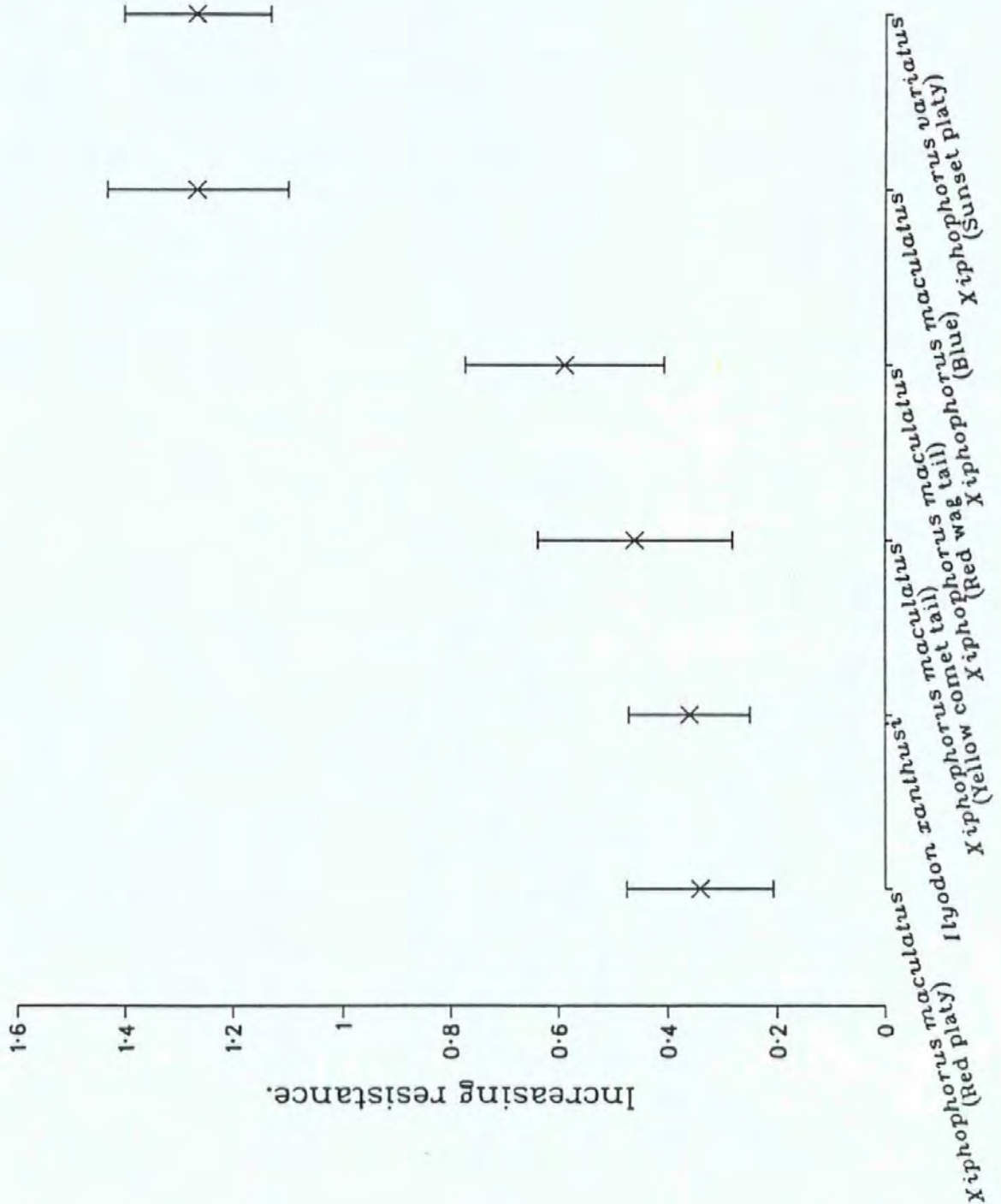
Significance levels as in Table 4.

**Table 7** Pairwise comparisons of strain and species differences in response to infection by *Icthyophthirius multifiliis*. The figure given above the diagonal is that of the estimate difference over standard error of that difference. Below the diagonal the significance level is given.<sup>5</sup>

|  | <i>Ameca splendens</i> | <i>Ilyodon xanthusi</i> | <i>Xiphophorus maculatus</i> (Yellow comet tail platy) | <i>Xiphophorus maculatus</i> (Red platy) | <i>Xiphophorus maculatus</i> (Blue platy) | <i>Xiphophorus maculatus</i> (Red wag tail platy) | <i>Xiphophorus variatus</i> (Sunset platy) |
|--|------------------------|-------------------------|--|--|---|---|--|
| <i>Ameca splendens</i>                                 |                        | 3.25                    | 2.57   | 2.56                                     | 7.60                                      | 3.22  | 9.34                                       |
| <i>Ilyodon xanthusi</i>                                | ***                    |                         | 0.53   | 0.13                                     | 5.24                                      | 1.22  | 6.52                                       |
| <i>Xiphophorus maculatus</i> (Yellow comet tail platy) | **                     | NS                      |  | 0.64                                     | 4.23                                      | 0.65  | 4.56                                       |
| <i>Xiphophorus maculatus</i> (Red platy)               | **                     | NS                      | NS   |  | 5.26                                      | 1.33  | 6.34                                       |
| <i>Xiphophorus maculatus</i> (Blue platy)              | ****                   | ****                    | ****   | ****                                     |   | 4.12  | 0.01                                       |
| <i>Xiphophorus maculatus</i> (Red wag tail platy)      | ***                    | NS                      | NS   | NS                                       | ****                                      |   | 4.52                                       |
| <i>Xiphophorus variatus</i> (Sunset platy)             | ****                   | ****                    | ****   | ****                                     | NS  | ****  |  |

<sup>5</sup>Significance levels as in Table 4.

Figure 23 Inter- and intra- specific differences in resistance to ichthyophthiriasis following analysis of all factors. Values are displayed on a relative scale of increasing resistance, relative to *Ameba splendens* with a resistance of zero.



## Chapter 5

### Between genotype comparisons: the common carp, *Cyprinus carpio*.

#### 5.1. Introduction and aims.

The pleiotropic effect of the scale pattern genes and the genetics of the carp used in this study were discussed in chapter 2. The main points from that discussion are re-iterated here. The genetics of scale pattern determination in carp was first described in 1928 by Rudzinski (Kirpichnikov, 1981). Two pairs of autosomal, unlinked genes control scale pattern cover in carp. Four scale pattern types exist; fully scaled, scattered mirror, linear mirror and leather carp (see Figures 14 to 17). The two gene pairs are denoted by *S* and *N*, with the dominant homozygous *NN* state being lethal. These genes have been shown to have a pleiotropic effect on several morphological and physiological characters, including two cases of disease resistance. The decreased fitness of *N* allele carp is common throughout these studies. The *S* locus also has a pleiotropic effect, but one which is less pronounced. Several studies have shown that in the presence of the *N* allele the action of the *S* allele is greatly increased (Kirpichnikov, 1981).

It is the aim of this investigation to evaluate the resistance of known genotypes of the four scale patterns of common carp to infection by *Icthyophthirius multifiliis*. These four scale types will differ only at their *S* and *N* loci, all other loci being equally distributed in the population. Hence, differences between scale types can be attributed to these loci. Furthermore, common carp are a species of commercial interest and hence any differences in resistance found here have direct implications for aquaculture.

## 5.2. Materials and methods.

### 5.2.1. Experimental design.

In an attempt to make the results from this investigation applicable to a wide range of environmental situations a nested experimental design was used (Figure 24). Two 'strains' of parasite were used. Other parts of this study used different isolates of parasite, which were all obtained from recently imported tropical fish. This investigation however, used several batches of parasite from British coldwater fish as well as several batches from tropical species imported from Singapore. Hence 'strains' of parasite from different continents could be compared. The British 'strain' was termed the temperate strain and the 'strain' imported from Singapore the tropical strain. The temperate strain was passaged at 14-16°C and the tropical strain at 23-25°C.

In comparing carp of each of the four scale pattern phenotypes it is important to be certain that all genes, except the *S* and *N* genes for scale pattern, are randomly distributed throughout the four groups. Hence, an ideal situation is to use the progeny from a single batch of eggs which, when raised, produces all four phenotypes. Some difficulty was experienced in obtaining such a group of full-sibs, since it is not considered economically worthwhile to produce fully scaled carp and leather carp, due to a lack of demand for such fish. The vast majority of carp farms in the U.K. therefore only produce scattered mirror and linear mirror carp. The carp used here were obtained from Hampshire Carp Hatcheries as 4-6 week old fry. These fish were obtained as two half-sib groups. The common maternal fish was a scattered mirror carp. Crossing this with a linear mirror carp produced clearly recognisable scattered mirror, linear mirror and leather carp in roughly equal proportions. In theory fully scaled carp should also be produced from this cross (Table 2), but raising these fry proved the suppliers prediction of all scale types except fully scaled to be correct. Hence genotypes were deduced to be : scattered mirror carp - *ssnn*, linear mirror carp - *SsNn*, and leather carp - *ssNn*. To obtain some fully scaled carp fry the same female, scattered mirror carp was crossed with a male, fully scaled

carp. The offspring from this cross were 100% fully scaled carp and hence (Table 2) were of the genotype *Ssnn*. The fry were raised on a 3,000 gallon closed recirculation system within the Polytechnic. Initially the fry were fed freshly hatched *Artemia* nauplii, but these were gradually replaced with trout pellets of appropriate size as the fish grew.

For each scale type of carp experiments were performed at three different temperatures ; 24°C, 19°C and 16°C (Figure 24 ). Carp were acclimatised to these temperatures for a minimum period of one month. However, the fish had usually been acclimatized for a period of time much greater than this. Fish, of all scale types, measured  $3.8 \pm 0.7$ cm in total length and  $2.9 \pm 0.5$ cm in standard length.

Within each temperature each of the four scale types of common carp were infected, with either the tropical or the temperate strain of the parasite. The exception was at 19°C where, due to lack of numbers of fish, the linear mirror carp were not infected. At 16°C 47 fish were infected, at 19°C 88 fish were infected and at 24°C 82 fish were infected. The carp were 42 - 56 weeks old when infected with *I. multifiliis*.

To determine the extent of any tank effects replicate infections were performed on three occasions. Fully scaled carp were used for these replicate procedures, since greater numbers of this scale type were available. On each occasion two tanks of ten fish were infected with the same batch of theronts, from the same parasite strain and at the same temperature. One replicate was performed at each temperature, with the tropical parasite strain being used at 24°C and 16°C and the temperate parasite strain being used at 19°C. Preliminary experiments suggested that carp were more resistant than *A. splendens* and that 4000 theronts per carp would produce levels of infection on the fish suitable for analysis. Infections were carried out therefore using 4000 theronts per fish (80 theronts per ml) and the response was measured as in chapter 3. Water quality was monitored prior to and upon termination of the experiment.



### 5.2.2. Analysis.

The Generalised Linear Interactive Modelling package (GLIM) was used on a Prime computer to analyse the parasite burdens on the fish. Factors included in this analysis were time of the experiment, temperature of the experiment, surface area of the fish, strain of parasite, and scale pattern phenotype. The analysis is based on the assumption that the variance and the means are independent. This is equivalent to the residual and fitted values being independent. In the case of the zero values occurring in the data set the normal practice of taking the logarithmic value of  $(y+1)$  was performed on all counts.

### 5.3. Results.

A total of 217 fish were successfully infected with *I. multifiliis* (106 fully scaled carp, 46 scattered mirror carp, 31 linear mirror carp and 34 leather carp). The surface areas over which infection levels were counted were very similar for each scale type : fully scaled carp  $4.02 \pm 1.50$  cm<sup>2</sup>, scattered mirror carp,  $3.77 \pm 1.41$  cm<sup>2</sup>, linear mirror carp,  $4.16 \pm 1.90$  cm<sup>2</sup> and leather carp,  $3.33 \pm 1.14$  cm<sup>2</sup>. Total counts of parasite burdens per fish ranged from 0 to 329 (mean =  $25.2 \pm 47.3$ ). Irrespective of parasite strain and temperature of the experiment, mean parasite burdens were : fully scaled carp  $13.44 \pm 26.55$  trophozoites per fish, scattered mirror carp  $35.98 \pm 61.71$  trophozoites per fish, linear mirror carp  $26.13 \pm 40.45$  trophozoites per fish, and leather carp  $46.40 \pm 68.20$  trophozoites per fish. These values are broken down further for each parasite strain and temperature in Table 8. On the three occasions when replicate infections were performed, on fully scaled carp, tank effects were not observed (*t*-tests,  $P > 0.10$ ).

The infections occurred at six different points in time, over a period of some four months. Seven infections, in addition to these, were unsuccessful. Carp from the latter infections exhibited no parasites, in the majority of cases, and less than five parasites in the few remaining cases. The data from these experiments was not utilized in the analysis and these fish were not used for any re-infections. Out of the six successful infections, fully scaled carp were

infected at each time, scattered mirror carp at five of the six times and linear mirror and leather carp at four of the six times. Each scale pattern phenotype was separately infected on at least one occasion with each of the parasite strains. In three of the six successful infections, both strains of parasite were used (on different groups of fish), to facilitate parasite strain comparisons. Of the 217 fish which were successfully infected, 131 were infected with the tropical strain of *I. multifiliis* and 86 were infected with the temperate strain of *I. multifiliis*. Parasite burdens on all scale pattern phenotypes and at all temperatures ranged from 0 to 179 for the tropical parasite strain infections and from 0 to 329 for the temperate parasite strain infections. At each of the three temperature regimes temperatures were relatively constant over the course of the experiment ( $16.01 \pm 0.81$  °C,  $19.34 \pm 0.37$  °C,  $23.85 \pm 1.07$  °C).

### 5.3.1. Analysis.

Figure 25 shows plots of residual values against fitted values for untransformed, square root transformed and logarithmic transformed data. From the clearly non-random distribution of untransformed data there is a progression to a random distribution in the case of the logarithmic transformed data. Hence, logarithmic transformed data fulfils the assumptions of analysis of variance and is the data set used in the following analysis, which is shown in Table 9.

Taking major effects first of all, only parasite strain effects are not significant. Effects due to temperature are significant at the 5% level, whilst all other effects are significant at the 0.1% level (*i.e.* surface area, time of experiment and scale type). Although carp with larger surface areas harboured more parasites, the relationship was not a direct one, *i.e.* a doubling of surface area does not imply a doubling of the parasite burden. The GLIM package estimated that a 100% increase in surface area would be accompanied by a  $30 \pm 5\%$  increase in infection level. This relationship was significant ( $P < 0.001$ ) and covered the range in surface areas from 1.20 to 9.30 cm<sup>2</sup>. Therefore, this stresses the need to separate this variation when determining the variation in resistance to *I. multifiliis* infection in stocks of fish.

Only a single parasite isolate was used for each strain of parasite (strains K and L, Appendix B). Differences in the infectivity of the two strains were not significant ( $P > 0.10$ ), but temporal variation in infection levels was seen for both strains of the parasite (Figure 27). Infections were attempted during April five times, but were unsuccessful. During May however the majority (64%) of fish were infected and the highest infection levels for both parasite strains were recorded (temperate strain, 329 trophozoites per fish, tropical strain 179 trophozoites per fish). Following this another unsuccessful infection occurred. The next infection occurred in the second half of June, with infection levels of upto 58 trophozoites per fish. Following this insufficient theronts precluded infection attempts until early August. Here a single unsuccessful infection was followed by a successful infection, but only with infection levels of upto 13 trophozoites per fish. No further infections were attempted after this time, despite the strains remaining in passage at a low level for a further 64 and 121 days for strains K and L respectively.

Further analysis of the scale type effects, using pairwise comparisons, demonstrates that the four scale patterns can be divided into two groups; (a) fully scaled carp and (b) scattered mirror, linear mirror and leather carp. The difference between these two groups is significant at the 0.1% level. The first of these groups has a mean infection level of  $13.44 \pm 25.55$  trophozoites per fish, whilst the second group has a mean infection level of  $36.41 \pm 58.84$  trophozoites per fish. Hence significant between genotype differences were observed with fully scaled carp being more resistant than the other three genotypes.

Three interaction terms were also analysed; scale type-parasite strain effects, scale type-temperature effects and parasite strain-temperature effects. Due to the data not being strictly orthogonal this had a slight, but not marked, effect upon the distributions of residual vs fitted value plots (Figure 26). These interaction terms provide some very interesting significant effects. Fully scaled, scattered mirror and leather carp all responded to the two parasite strains in a similar manner ( $P < 0.10$ ), but linear mirror carp differed from the other three scale types at the 0.1% level. Figure 28 shows this response to each parasite

strain for all of the four scale pattern phenotypes. It can be seen that for each scale type, except linear mirror carp, infection levels were higher for the temperate strain than the tropical parasite strain. However, the data for the linear mirror carp is composed of the smallest number of points, and those for the temperate strain only number seven, from a single tank of infected fish. Therefore, this apparent difference in the case of linear mirror carp is quite possibly due to insufficient data. The significance of this effect should therefore be treated with caution.

With respect to scale-temperature interactions, pairwise comparisons were not significant ( $P > 0.10$ ), in every case except for that between fully scaled carp and scattered mirror carp ( $P < 0.05$ ). Figure 29 shows the relationship between temperature and infection level for each scale type of *C. carpio*. It can be seen that there is a greater reduction in infection levels with increasing temperature for fully scaled carp than there is for scattered mirror carp. Hence, the difference between the two scale types is greater at higher temperatures.

Whilst the variation in infection levels due to the use of different parasite strains was not significant, the two parasite strains did differ in their responses to changing temperature ( $P < 0.10$ ). Figure 30 shows the change in infection levels with temperature for each parasite strain. It will be recalled that there are 131 data points for the tropical parasite strain and 86 data points for the temperate parasite strain. The two distributions are distinctly different. Infection levels increase with increasing temperature for the tropical parasite strain, whilst they *decrease* with increasing temperatures for the temperate parasite strain. Infection levels also increased to higher levels with the temperate parasite strain (maximum = 329 trophozoites per fish) compared to the tropical parasite strain (maximum = 179 trophozoites per fish). The range of temperatures over which data was recorded was 9.8°C for the tropical parasite strain and 5.9°C for the temperate parasite strain. Parasite strains therefore do differ in their behaviour at different temperatures.

## 5.4. Discussion.

In the previous chapter the presence of genetic variation between species and between strains within species, for resistance to *I. multifiliis* infections was established. A similar approach was taken here, but the experimental design was such that a lot more detailed information was obtained. The common carp, *C. carpio*, was chosen, since this is a species which is cultured throughout the world and which has been the subject of many genetical studies. In order that the conclusions of this study would be applicable to a wide variety of environmental situations two 'strains' of parasite and three temperature regimes were employed.

Once again, effects due to the time at which the experiment was performed were quite marked. A plot of infection levels against time, for successful and unsuccessful infections, shows this annual distribution (Figure 27). This annual distribution is far more complex than that of the previous chapter, since infections here also occurred at three different temperatures. However, when similar temperature regimes were used for both parasite strains at the same point in time, infection levels followed the same trend. Although data was recorded only over a period of four months the distribution again matches with that found in natural ponds. Infections were unsuccessful during April, but during May large infection levels (upto 329 trophozoites per fish) were recorded. By late June insufficient parasite was available to even attempt infections. Early August saw infections once more, but only at a low level (upto 13 trophozoites per fish). Following this the parasite could only be passaged at very low levels until the two strains were lost in early October and December. This interesting annual distribution will be discussed further in the final chapter.

The range of temperatures used here was much greater than previous, being approximately 10°C. Significant variation due to temperature was demonstrated. It can be seen from Figure 30 that infection levels increased with temperature for the tropical parasite strain. A similar effect was seen for within and between species comparisons in the previous chapter. The point was made there that temperatures also encode within them tank effects. On

three occasions here more than one tank of fully scaled carp were infected simultaneously and at the same temperature and with the same parasite strain. Such tanks did not differ (*t*-test,  $P > 0.10$ ), suggesting that tank effects here were minimal, at least for fully scaled carp at each of the three temperatures and for each parasite strain. Counts of parasites on fish were quite low at lower temperatures for the tropical parasite strain (16°C,  $9.38 \pm 10.90$  trophozoites per fish, 19°C,  $17.13 \pm 32.70$  trophozoites per fish, 24°C,  $14.45 \pm 32.48$  trophozoites per fish). This has the consequence that in colder climates there is less variation in infection levels, thus reducing the selection differential which would be obtainable in any selection programme. However, for the temperate parasite strain the opposite is true - 16°C, no infections; 19°C,  $63.12 \pm 70.25$  trophozoites per fish, 24°C,  $4.48 \pm 3.46$  trophozoites per fish. Hence, the level of infection at a particular temperature depends upon which parasite strain caused the infection. Similarly, the effect of a changing temperature may promote or inhibit an *I. multifiliis* epizootic, depending upon the parasite strain present.

The range of surface area values here (1.2 - 9.3 cm<sup>2</sup>) was similar to that in the previous chapter (1.5 - 11.1 cm<sup>2</sup>) and once again larger fish were found to harbour relatively less parasites. Young carp grow quickly and, therefore, in a disease study such as this, it is essential to determine the surface area of the infected fish in every infection.

The most interesting points of this investigation are the comparisons of different scale types and the comparison of different parasite strains. Fully scaled carp were found to differ from the other three scale types, which formed a homologous group. Scale pattern genotypes for the four scale types were; fully scaled carp, *Ssnn*; scattered mirror carp, *ssnn*; linear mirror carp, *SsNn*; and leather carp, *ssNn*. The *t* statistic, used to determine the statistical significance of differences among scale types, showed that there was no significant difference among the three genotypes *ssnn*, *SsNn* and *ssNn*. One comparison made by other workers (Probst, 1953; Chan May-Tchien, 1969; Kirpichnikov, 1981) is to observe the differences between the two groups of carp with *Nn* and *nn* genotypes. Here this amounts to comparing linear mirror

and leather carp with fully scaled and scattered mirror carp. Similarly *Ss* fish can be compared with *ss* fish by observing the differences between fully scaled and linear mirror carp, as one group, and scattered mirror and leather carp, as the second group. Such comparisons are not meaningful here though since fully scaled carp were found to differ significantly from the other three scale pattern phenotypes, which formed an homologous group.

It is widely accepted that the *N* allele has the action of reducing fitness in carp (Kirpichnikov *et al*, 1937). Probst (1953) suggested that the *N* gene is associated with a defect in the development of the mesenchyme. Kirpichnikov (1981) considered the *N* allele to represent a large mutation, probably involving a chromosomal rearrangement and deletion of a small part of the chromosome. The action of the *S* allele is less well documented, but the homozygous dominant state is considered to have been the ancestral type (Kirpichnikov, 1981). The mutation of the *S* allele to the *s* allele then resulted in the appearance of mirror carp, with a reduced fitness (Kirpichnikov, 1981). Hence, it can be said that the presence of the *N* allele reduces fitness, whilst the *S* allele promotes fitness.

The ranking of the four scale types from most to least resistant is as follows : fully scaled carp (*Ssnn*),  $13.44 \pm 26.55$  trophozoites per fish; linear mirror carp (*SsNn*),  $26.13 \pm 40.45$  trophozoites per fish; scattered mirror carp (*ssnn*)  $35.98 \pm 61.71$  trophozoites per fish; and leather carp (*ssNn*)  $46.40 \pm 68.20$  trophozoites per fish. Hence it can be seen that the results here agree with the notion of the *S* allele promoting resistance and the *N* allele reducing resistance. An explanation of why the *Ssnn* genotype is significantly more resistant than the group of *SsNn*, *ssn* and *ssNn* genotypes, requires the hypothesis that the action of the *S* allele is greater in the absence of the *N* allele. Kirpichnikov (1981) however states that in the *presence* of the *N* allele the action of the *S* allele is stronger. The action upon the reduction of all the fins is cited as an example, but the explanation of the detail of this is confusing (leather and linear mirror carp genotypes are only referred to as *s,N* and *S,N* respectively) and the original references are written in Russian.

Analysis of scale type-parasite strain interactions demonstrated highly significant differences between linear mirror carp and the other scale types. However, it was pointed out that this significance was quite likely to be due to the lack of data for linear mirror carp infected with the temperate parasite strain (only a single tank of seven fish were infected). Fully scaled carp differed only from scattered mirror carp in terms of scale type-temperature interactions; but fully scaled carp were not significantly different from either linear mirror or leather carp. Therefore, there is some degree of similarity between the response of fully scaled carp, linear mirror and leather carp with respect to changing temperatures and different strains of parasite. However, whilst the pattern of response to these parameters is similar, the level at which that response is occurring is different, as shown by the significant scale type differences.

Differences between *Nn* and *nn* fish are, according to Kirpichnikov (1981), more apparent under unfavourable conditions. The apparent similarity of scattered mirror carp (*ssnn*) with linear mirror carp (*SsNn*) and leather carp (*ssNn*) here could, therefore, be due to favourable conditions being present for all scale types. Such favourable conditions include water quality and stocking levels. Here water quality was kept at an optimum level and stocking densities maintained as low as possible. It must be borne in mind that under different conditions further differences between scale types may become apparent.

The consequences of fully scaled carp being the most resistant group depends upon where those carp are being cultured. In general fully scaled carp are not cultured as edible fish since mirror carp have fewer scales, making food preparation easier. Kirpichnikov (1981) states that leather carp are not produced in the U.S.S.R., the F.R.G., the G.D.R. and a number of other countries. Enquiries around the U.K., in order to obtain the fish used here, showed that production is predominately of scattered mirror carp, except for ornamental purposes. Production in Israel is apparently of scattered mirror carp also (Hines & Spira, 1973b).



Finally, it was interesting that effects due to parasite strains were not significant. There has been some discussion for a number of years as to whether different strains of *I. multifiliis* exist. The two parasite strains here arose from different continents and whilst comparisons of infection levels for the two parasite strains were not significant, their responses to changing temperatures were significantly different. There are two possible explanations for this; i. genotype-environment differences between strains exist, or ii. the difference in response is a reflection of the temperature to which the parasite strain was acclimated. In an attempt to determine which of these two explanations was correct it was repeatedly attempted to acclimatise the temperate strain from 16°C to 24°C and the tropical strain from 24°C to 16°C. This was initially attempted as a one step process and then, later, as a two step process. Should a similar effect still be present with the strains in a reversed mode of passage then parasite strain-temperature interactions were likely to be due to different acclimation temperatures.

However, in the majority of cases strains of parasite could not be moved to a different temperature regime for more than a single passage. Indeed, the few infections which survived more than one passage at the new temperature produced only a very small number of theronts and soon died out. One can only assume therefore, that each parasite strain is adapted to its acclimation temperature and will not tolerate deviations of 8°C from this. Further information in support of this is that 'granular' areas were often seen on fish infected at the opposite extreme of temperature to the parasites acclimation temperature (*i.e.* 24°C for the temperate strain and 16°C for the tropical strain). Nothing conclusive can be said about these 'granular' areas, but they have the appearance and distribution of previous parasite locations. The area was not open to the exterior, as if the parasite had left the host, but was covered over and the contents suggested debris left from the remains of a parasite. Presumably some host response or environmental factor had resulted in the parasites demise and degradation. Further work, attempting to acclimatise the parasite to different temperatures in smaller steps, is required before discussing this matter in greater depth.

It is proposed therefore that parasite strain differences do exist, although conclusive proof is still lacking. The response to temperature changes from the parasites norm is interesting in that one recommended treatment for ichthyophthiriasis is to increase the temperature. For the temperate strain of parasite used here increasing the temperature to over 26°C would eradicate the parasite, Figure 30. Such an action would, therefore, reduce the number of theronts produced in successive parasite cycles and also possibly promote the destruction of parasites *in situ* on the host. The latter point merits further investigation since only one chemical, mercurous acetate, is known to have any effect on *I. multifiliis* parasites *in situ* (Dashu & Lien-Siang, 1960; Nousias, 1987) and its' use on food fish is questionable. However, raising the temperature for infections with the tropical parasite strain would only serve to increase infection levels, Figure 30. This is perhaps the reason for the debate over the effectiveness of increased temperature upon the control of ichthyophthiriasis.

In summary then, genetic differences do exist between different carp scale phenotypes and possibly between strains of parasite. However, the most resistant scale type (fully scaled) is not the most commonly cultured scale type. Environmental conditions have been shown to affect resistance to ichthyophthiriasis, in terms of temperature. Hence differences between scale types will be more apparent in particular climates. Should scale types be improved further then the techniques of selection and/or hybridization must be employed. The effectiveness of such approaches in increasing resistance to ichthyophthiriasis will be investigated in the next two chapters.

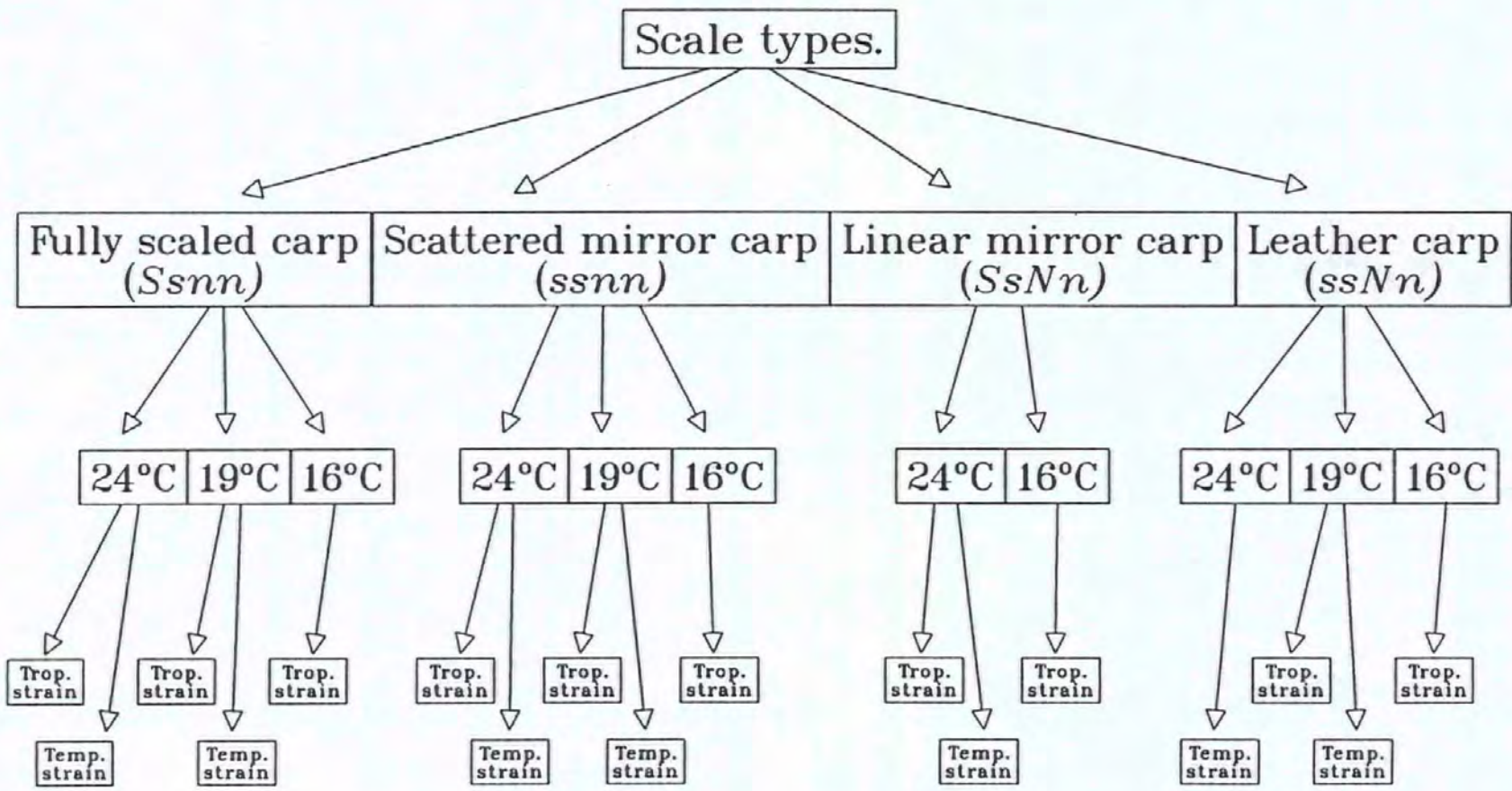
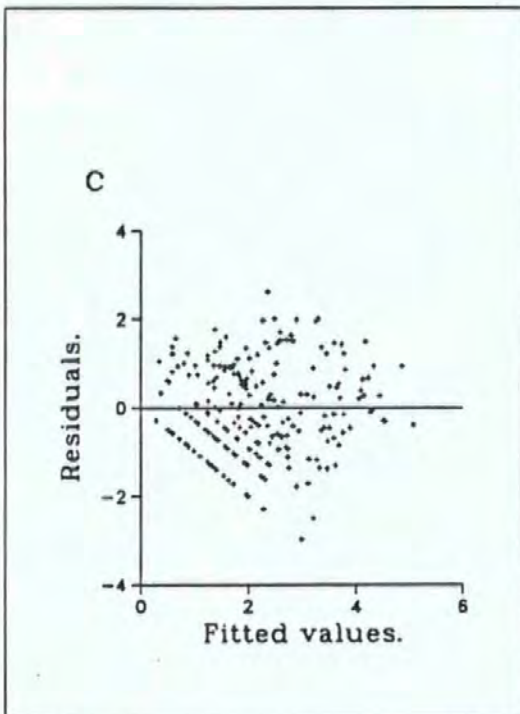
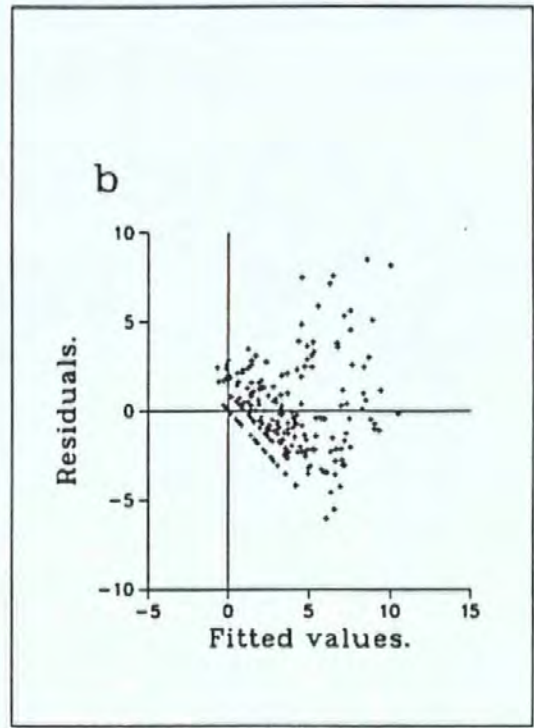
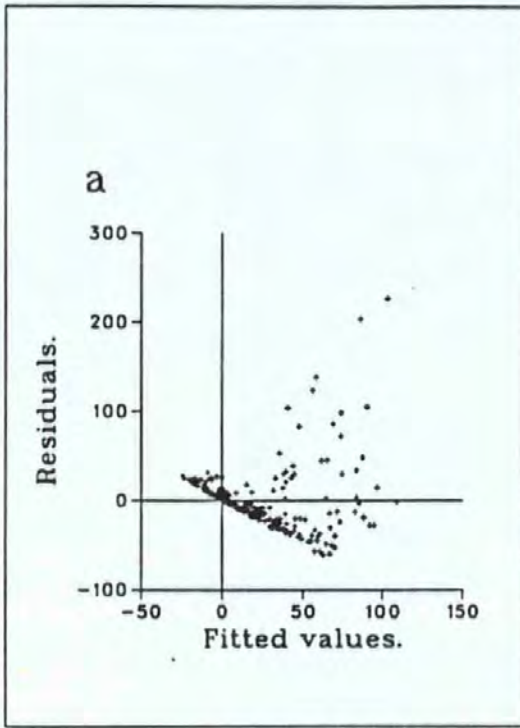


Figure 24 Nested experimental design used to assess the resistance of *Cyprinus carpio* to infection by *Icthyophthirius multifiliis*.

|                                   | Fully scaled carp. | Scattered mirror carp. | Linear mirror carp. | Leather carp.      | Total n |
|-----------------------------------|--------------------|------------------------|---------------------|--------------------|---------|
| <b>Tropical parasite strain.</b>  |                    |                        |                     |                    |         |
| 16°C                              | 10.55 ± 6.46 (20)  | 4.58 ± 8.14 (12)       | 19.57 ± 21.14 (7)   | 4.75 ± 3.01 (8)    | 47      |
| 19°C                              | 3.12 ± 3.06 (17)   | 32.14 ± 35.90 (7)      | ---                 | 36.14 ± 52.85 (7)  | 31      |
| 24°C                              | 1.93 ± 2.45 (29)   | 10.14 ± 7.15 (7)       | 37.59 ± 50.37 (17)  | ---                | 53      |
| Total n                           | 66                 | 26                     | 24                  | 15                 | 131     |
| <b>Temperate parasite strain.</b> |                    |                        |                     |                    |         |
| 16°C                              | ---                | ---                    | ---                 | ---                | 0       |
| 19°C                              | 35.40 ± 42.23 (30) | 91.14 ± 86.58 (14)     | ---                 | 63.12 ± 70.25 (13) | 57      |
| 24°C                              | 4.30 ± 3.33 (10)   | 4.67 ± 3.14 (6)        | 4.86 ± 4.30 (7)     | 4.48 ± 3.46 (6)    | 29      |
| Total n                           | 40                 | 20                     | 7                   | 19                 | 86      |

**Table 8** *Ichthyophthirius multifiliis* levels on the four scale pattern types of common carp, *Cyprinus carpio*, infected with two parasite strains at three temperature regimes.

Figure 25 Plots of residual values vs fitted values after analysis for the parasite burdens of *Icthyophthirius multifiliis* on the four scale types of common carp (*Cyprinus carpio*).  
a. Untransformed data, b. Square root transformed data, c. Natural logarithmic transformed data.



**Table 9** Analysis of variance tables for each of the variables in *Icthyophthirius multifiliis* infections of the four scale types of common carp, *Cyprinus carpio*.<sup>6</sup>

| Source of variation.  | d.f. | Sum of Squares. | Mean Squares. | F    | P    |
|---|------|-----------------|---------------|------|------|
| Effects due to area, scale type, parasite strain and temperature. | 6    | 137.4           | 22.9          | 21.7 | **** |
| Effects due to time.  | 5    | 91.8            | 18.4          | 17.4 | **** |
| Residual.   | 205  | 216.6           | 1.1           |      |      |
| Total.  | 216  | 445.8           |               |      |      |
| Effects due to time, scale type, parasite strain and temperature. | 10   | 188.9           | 18.9          | 17.9 | **** |
| Effects due to area.  | 1    | 40.3            | 40.3          | 38.1 | **** |
| Residual.   | 205  | 216.6           | 1.1           |      |      |
| Total.  | 216  | 445.8           |               |      |      |
| Effects due to area, time, parasite strain and temperature.       | 8    | 194.5           | 24.3          | 23.0 | **** |
| Effects due to scale type.  | 3    | 34.7            | 11.6          | 10.9 | **** |
| Scale - parasite strain interactions.                             | 3    | 23.2            | 7.7           | 7.3  | **** |
| Scale - temperature interactions.                                 | 3    | 5.7             | 1.9           | 1.8  | NS   |
| Residual.   | 205  | 216.6           | 1.1           |      |      |
| Total.  | 216  | 445.8           |               |      |      |
| Effects due to area, time, scale type and temperature.            | 10   | 226.5           | 22.6          | 21.4 | **** |
| Effects due to parasite strain.                                   | 1    | 2.7             | 2.7           | 2.5  | NS   |
| Parasite strain - temperature interactions.                       | 1    | 4.0             | 4.0           | 3.8  | *    |
| Residual.   | 205  | 216.6           | 1.1           |      |      |
| Total.  | 216  | 445.8           |               |      |      |

<sup>6</sup>Significance levels as in Table 4.

**Table 9** continued ...

| Source of variation.                                       | <i>d.f.</i> | Sum of Squares. | Mean Squares. | <i>F</i> | <i>P</i> |
|--|-------------|-----------------|---------------|----------|----------|
| Effects due to area, time, scale type and parasite strain. | 10          | 222.8           | 22.3          | 21.1     | ****     |
| Effects due to temperature.                                | 1           | 6.4             | 6.4           | 6.1      | **       |
| Residual.  | 205         | 216.6           | 1.1           |          |          |
| Total.   | 216         | 445.8           |               |          |          |

Significance levels as in Table 4.

Figure 26 Plots of residual values vs fitted values (log transformed data) after analysis of interaction effects of parasite burdens of *Icthyophthirius multifiliis* on the four scale types of common carp (*Cyprinus carpio*). a. Scale-parasite strain interactions, b. Parasite strain-temperature interactions, c. Scale-temperature interactions.

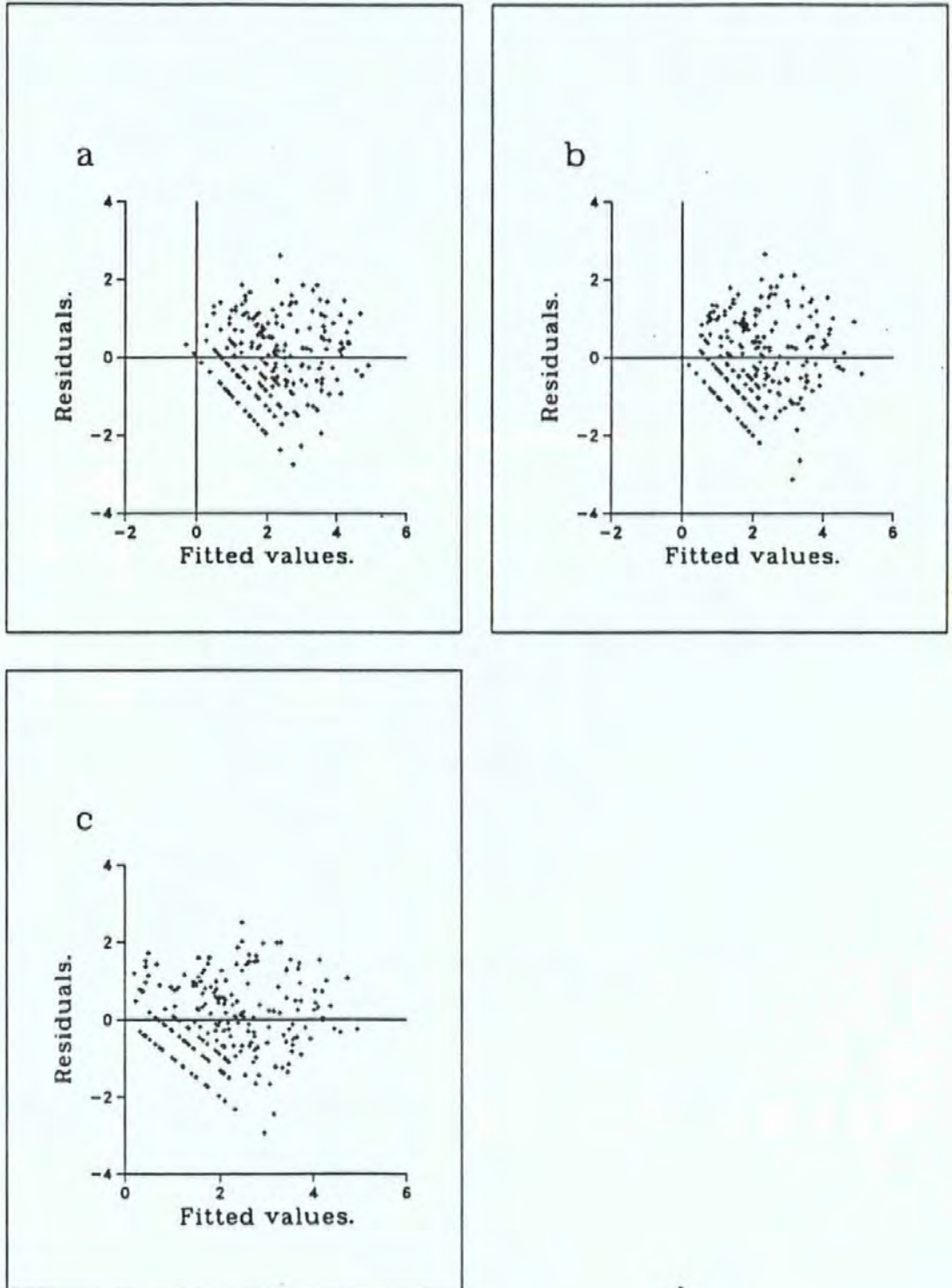




Figure 27 Annual variation in *Ichthyophthirius multifiliis* levels on the four scale types of common carp, *Cyprinus carpio*.

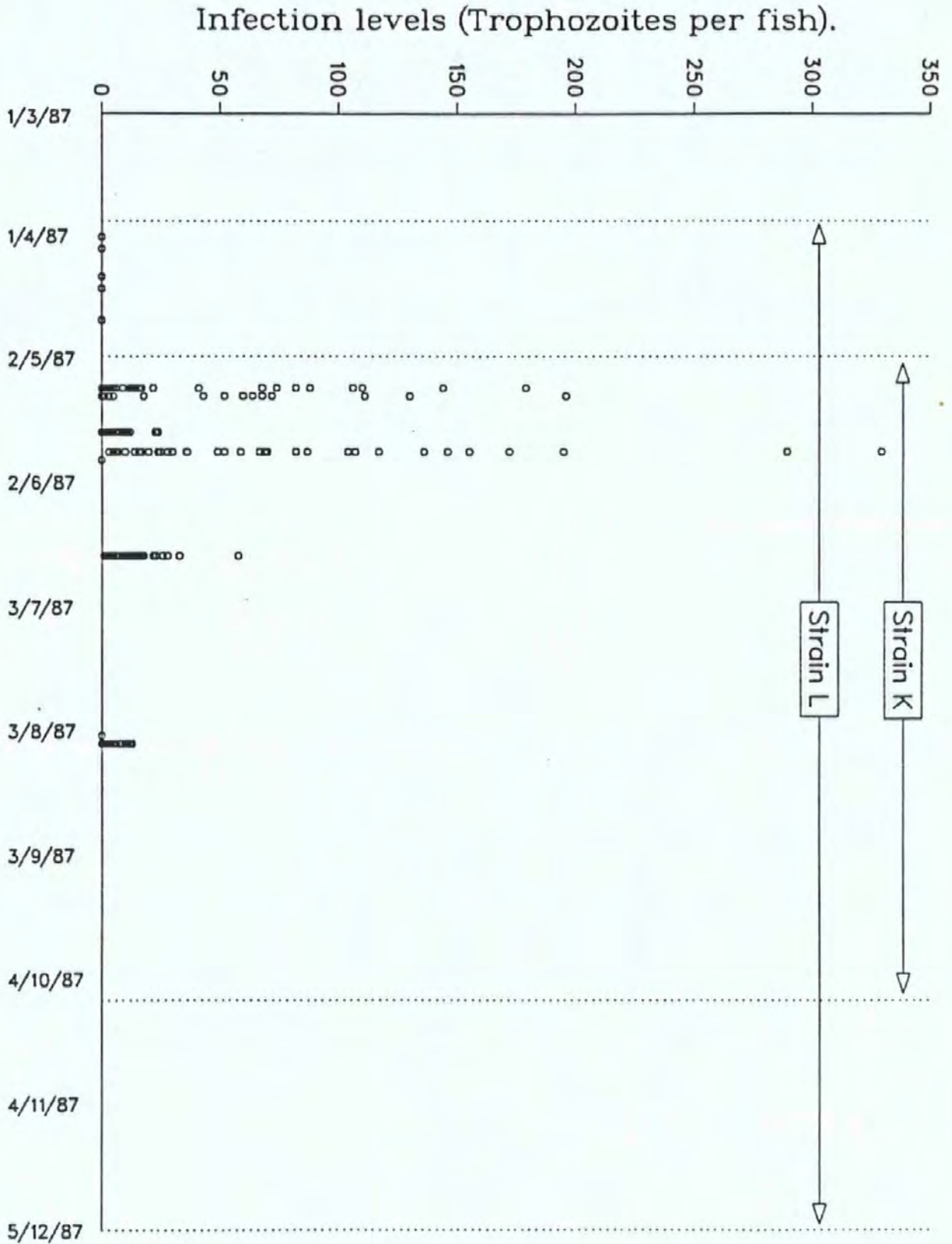


Figure 28 *Ichthyophthirius multifiliis* infection levels, for each parasite strain, on the four scale pattern phenotypes of the common carp, *Cyprinus carpio*. a. Fully scaled carp, b. Scattered mirror carp, c. Linear mirror carp, d. Leather carp.

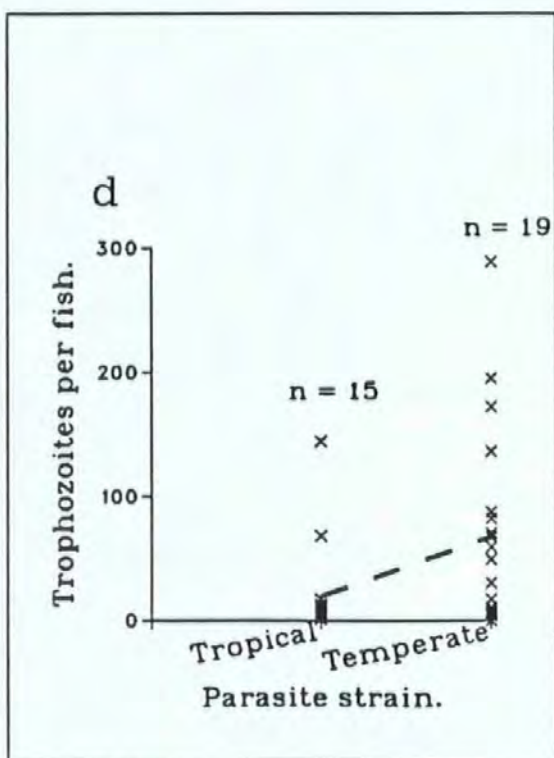
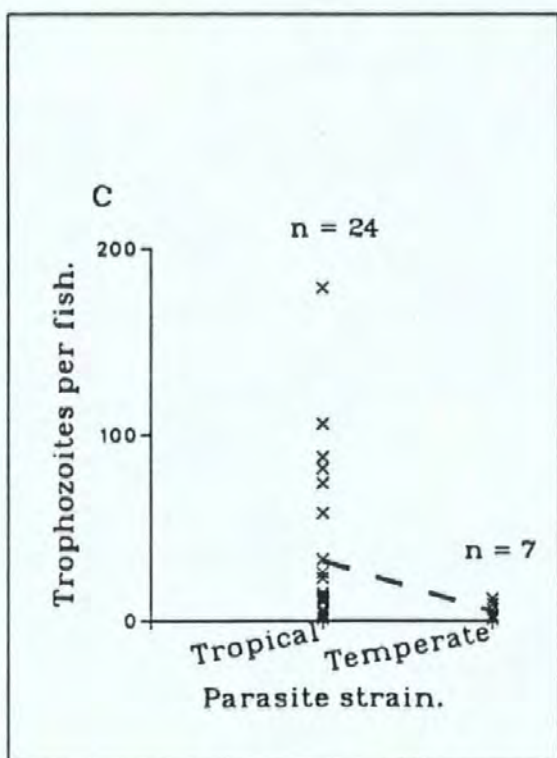
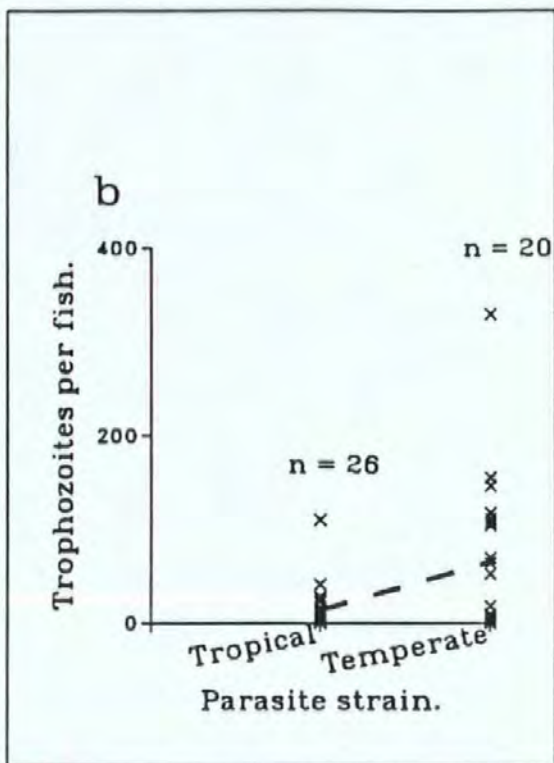
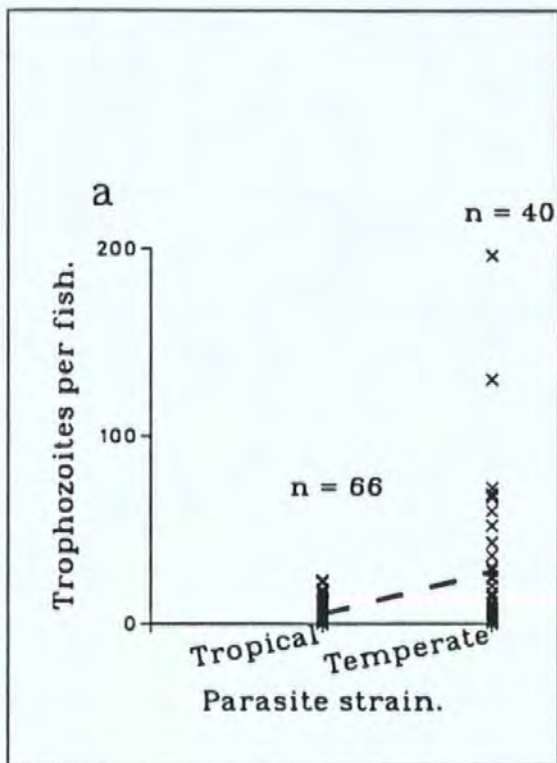


Figure 29 Variation in *Ichthyophthirius multifiliis* infection levels with temperature for each scale type of *Cyprinus carpio* and each parasite strain. a. Fully scaled carp, b. Scattered mirror carp, c. Linear mirror carp, d. Leather carp.

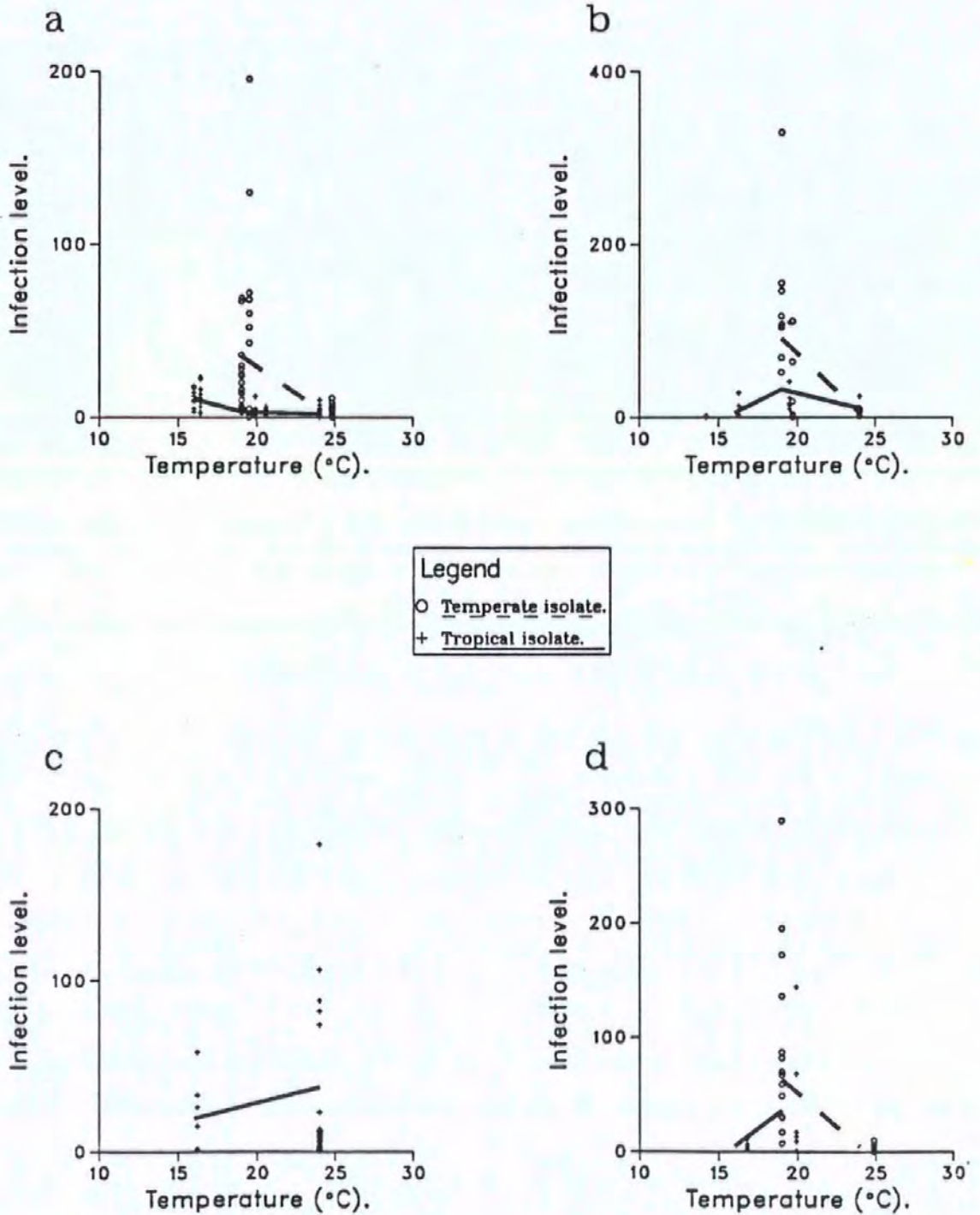
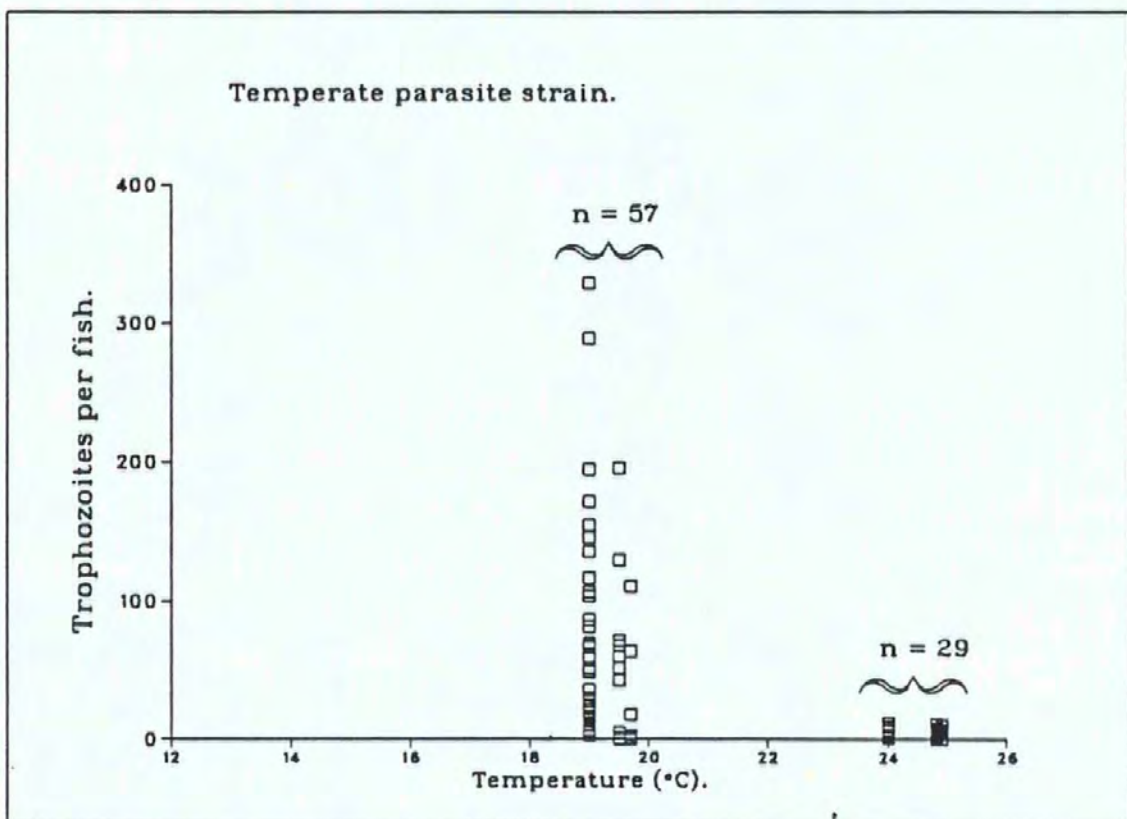
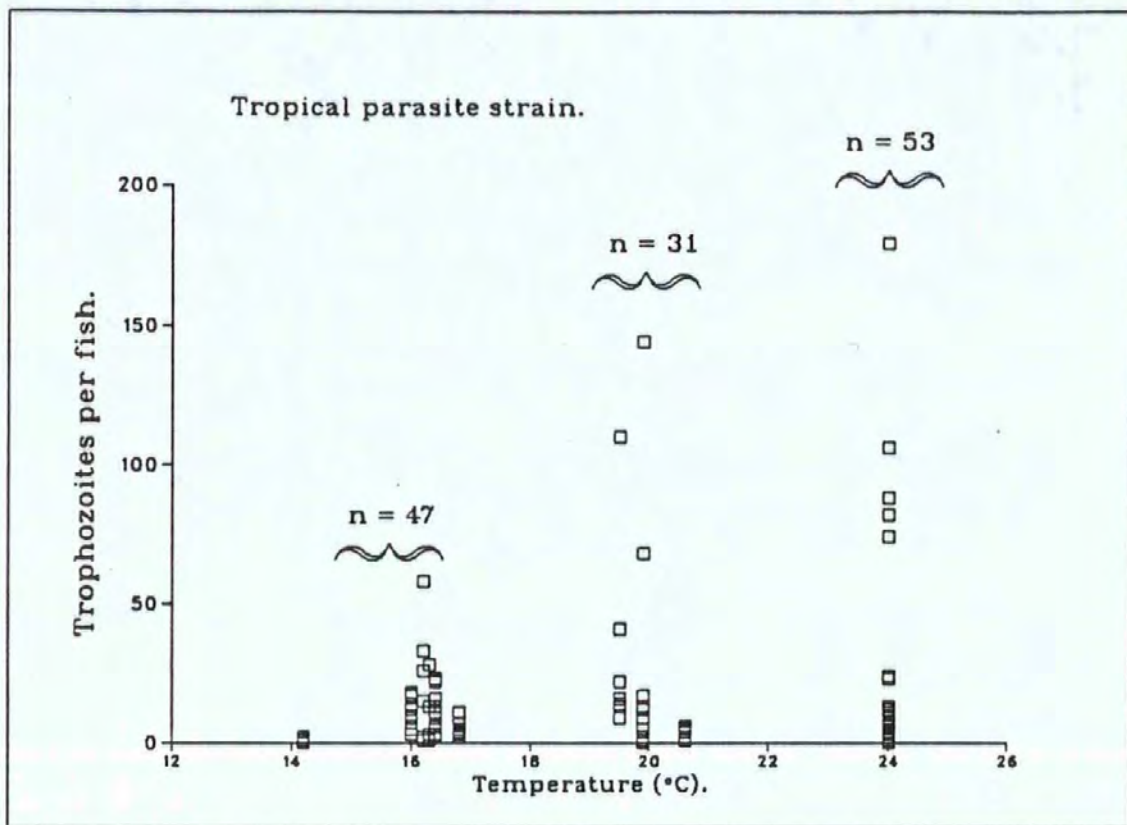


Figure 30 The relationship between temperature and infection level for each strain of *Icthyophthirius multifiliis*.



## Chapter 6

### Heritability estimation.

#### 6.1. Introduction.

The observed phenotypic variance ( $V_p$ ) of a trait can be partitioned into its causal components thus;

$$V_p = V_A + V_D + V_I + V_E$$

where;  $V_p$  = phenotypic variance,  $V_A$  = additive variance,  $V_D$  = dominance variance,  $V_I$  = interaction variance terms,  $V_E$  = environmental variance. Heritability ( $h^2$ ) is the ratio of additive variance to phenotypic variance of a trait ( $V_A/V_p$ ). Additive variance is the sum of the effect of each allele that contributes to the production of the phenotype, across all additive loci. Additive variance in a population does not depend upon specific allele interactions or combinations, and therefore is not disrupted during meiosis. Since additive variance is transmitted from one generation to the next, heritability is a useful quantity, allowing one to predict the following generations. For this reason, if the value of  $h^2$  is large, then it is worthwhile attempting to exploit the genetic variation through selection methods. Indeed, additive variance is considered by many as the greatest genetic resource available for stock improvement (Gall, 1983). The response ( $R$ ) to selection is simply the product of the heritability value ( $h^2$ ) and the selection differential ( $S$ ), where  $S$  is the size of the difference between the selected population and the population average. Heritability values lie between 0 and 1.0, and hence the greater the heritability value the larger the response ( $R$ ) to selection.

There have been many studies in aquaculture which have determined heritability values for a variety of traits. Gjedrem (1983) and Tave (1986) have perhaps the best summary tables of such studies to date. These studies

predominately concentrate on reproductive, morphological and other productive phenotypes. Studies involving fish pathogens are relatively few. Tolerance to *Vibrio anguillarum* in atlantic salmon has a heritability value of  $0.11 \pm 0.06$  (Gjedrem & Aulstad, 1974). In chum salmon Smoker (1981) obtained a heritability value of  $0.5 \pm 0.6$  for resistance to *Vibrio anguillarum*. McIntyre & Amend (1978) looked at resistance to IHN virus in sockeye salmon. Values of  $h^2$  varied between  $0.27 \pm 2.6$  and  $0.38 \pm 5.1$ . Bone (1983) found a heritability of zero for resistance to *Ichthyophthirius multifiliis* in one strain of platyfish (*Xiphophorus maculatus*, red wagtail).

Since the calculation of  $h^2$  involves  $V_p$ , then it also involves  $V_E$ , since  $V_E$  is a component of  $V_p$ . Several studies have shown that different environments change  $h^2$ , e.g. the heritability for hatching time was found to be 0 when rainbow trout eggs were hatched in incubators, but 0.23 when hatched in troughs (McIntyre & Blanc, 1973). Heritability values can thus only act as a guideline in a different environment to that in which it was measured. Moav & Wohlfarth (1976), for example, found a  $h^2 \approx 0$  for increased growth rate in an Israeli strain of *Cyprinus carpio*. As a result, many have concluded that it is impossible to improve productivity in common carp culture via selection, since little, or no, additive variance exists for increased growth rate. Smíšek (1979), however, found heritabilities of 0.49, 0.15, 0.25 and 0.21 for body weight at 1, 2, 3 and 4 years respectively in a Czechoslovakian population of carp.

## 6.2. Determination of heritability.

There are six methods of determining  $h^2$ , (Tave, 1986); full-sib analysis, half-sib analysis, parent-offspring analysis, mid-parent-offspring analysis, diallele analysis and realized  $h^2$  determination. The choice of analysis is determined by factors such as the numbers and types of relative available. Cattle breeding, for example, only produces a few offspring per female, but generally, large numbers of cows are mated. On the whole, however, Falconer (1981) suggests the use of the half-sib or regression of offspring on father methods, since the covariance in these two methods is least likely to be augmented by an environmental component.

The half-sib analysis is perhaps the most useful approach with fish. It draws a reasonable compromise between accuracy, amount of information obtainable and practicality. A number of males (sires) are each mated to several females (dams), the males and females being randomly chosen and assigned to each other. A number of offspring are then produced per dam and these provide the data in the form of a population of full-sibs and a population of half-sibs.

Becker (1975) describes the model for such a design and also the computation of the component parts.

$$Y_{ijk} = \mu + \alpha_i + \beta_{ij} + e_{ijk}$$

where  $Y_{ijk}$  is the record of the  $k^{th}$  progeny of the  $j^{th}$  dam, mated to the  $i^{th}$  sire;  $\mu$  is the common mean;  $\alpha_i$  is the effect of the  $i^{th}$  sire;  $\beta_{ij}$  is the effect of the  $j^{th}$  dam, mated to the  $i^{th}$  sire; and  $e_{ijk}$  is the uncontrolled environmental and genetic deviations attributed to the individuals.

An analysis of covariance is performed. The phenotypic variance can thus be partitioned into differences between the progeny of different males (the between sire component,  $\sigma_s^2$ ), differences between progeny mated to the same male (the between dam within sires component,  $\sigma_D^2$ ) and differences between individual offspring of the same female (the within progenies component,  $\sigma_W^2$ ).

The form of the analysis is therefore;

| <u>Source</u>                 | <u>d.f.</u> | <u>Mean square</u> | <u>Composition of mean square</u>                |
|-------------------------------|-------------|--------------------|--|
| Between sires.                | $s - 1$     | $MS_S$             | $= \sigma_W^2 + k_2 \sigma_D^2 + k_3 \sigma_s^2$ |
| Between dams<br>within sires. | $d - s$     | $MS_D$             | $= \sigma_W^2 + k_1 \sigma_D^2$                  |
| Within progenies.             | $n - d$     | $MS_W$             | $= \sigma_W^2$                                   |

where;

- $s$  = number of sires,
- $d$  = total number of dams,
- $n$  = total number of progeny,

and, for equal numbers of dams per sire and progeny per dam,

- $k_1 = k_2$  = number of progeny per dam,
- $k_3$  = number of progeny per sire.

From this it can be seen how each component can be calculated from the mean square values using the figures  $k_{1-3}$ . This, however, only applies to a balanced design. For unequal numbers of offspring per dam and dams per sire Dickerson (1959), Turner & Young (1969) and Becker (1975) have derived methods of estimating  $k_1, k_2, k_3, \sigma_S^2, \sigma_D^2$  and  $\sigma_W^2$ .

In terms of variance and covariance  $\sigma_S^2, \sigma_D^2$  and  $\sigma_W^2$  can be partitioned as below;

$$\begin{aligned} \text{Sires:} & \quad \sigma_S^2 = \text{Cov}_{(HS)} \\ \text{Dams:} & \quad \sigma_D^2 = \text{Cov}_{(FS)} - \text{Cov}_{(HS)} \\ \text{Progenies:} & \quad \sigma_W^2 = V_P - \text{Cov}_{(FS)} \\ \text{Total:} & \quad \sigma_T^2 = \sigma_S^2 + \sigma_D^2 + \sigma_W^2 = V_P \\ \text{Sires+Dams:} & \quad \sigma_S^2 + \sigma_D^2 = \text{Cov}_{(FS)} \end{aligned}$$

where,  $\text{Cov}_{(FS)}$  = covariance of full sibs and  $\text{Cov}_{(HS)}$  = covariance of half sibs. Hence, Becker (1975) demonstrated how  $h_S^2, h_D^2$  and  $h_{S+D}^2$ <sup>8</sup> can be deduced;

$$h_S^2 = \frac{4\sigma_S^2}{\sigma_T^2} = (V_A + \frac{1}{4}V_{AA} + \frac{1}{16}V_{AAA})/V_P$$

$$h_D^2 = \frac{4\sigma_D^2}{\sigma_T^2} = (V_A + V_D + \frac{3}{4}V_{AA} + \frac{1}{2}V_{AD} + \frac{1}{4}V_{DD} + \frac{7}{16}V_{AAA} + 4V_M)/V_P$$

$$h_{S+D}^2 = \frac{2(\sigma_S^2 + \sigma_D^2)}{\sigma_T^2} = (V_A + \frac{1}{2}V_D + \frac{1}{2}V_{AA} + \frac{1}{4}V_{AD} + \frac{1}{8}V_{DD} + \frac{1}{4}V_{AAA} + 2V_M)/V_P$$

Above  $V_I$  has been separated into its components to show where dominance interactions, in particular, are present. Maternal variation ( $V_M$ ) is an important element in the life of some animals.

Calculation of the standard error of each of these estimates is described by Turner & Young (1969) and Becker (1975).

<sup>8</sup>Heritability measured among sires, heritability measured among dams and heritability measured among sires and dams, respectively



### 6.3. Design of experiments and methodology.

Heritability studies were performed using a half-sib analysis approach. The species used were *X. maculatus* (blue platy), *X. maculatus* (red wag tail platy), *X. maculatus* (yellow comet tail platy), *X. maculatus* (Vera Cruz platy) and *Ameba splendens*. For each species five tanks were set up, each containing one male and four virgin females. None of these fish had ever been exposed to *I. multifiliis* previously. Plant cover (*Vesicularia dubyana*) was provided and the fish left to breed. Young from each female were isolated in a small perspex tank (2.4 l). Diet was initially fresh *Artemia* nauplii, followed by the addition of ground Promin pellets and ground Tetramin flakes. Water changes were performed regularly and fry were moved to 23.2 l tanks as they grew. When adult (approximately 1 year old) these offspring were then exposed to *I. multifiliis*. Infections were performed, as previously, using a dose of 2000 theronts per fish. Infection levels, which were counted after 3 days, were measured on an area basis (see chapter 3). An initial analysis was performed using the Generalised Linear Interactive Modelling package (GLIM) on a Prime computer (see chapter 3). For natural logarithmic transformations on data which included zero values the normal practice of using  $(y+1)$  was used. Following this, estimates of heritability were computed by the above method on a BBC microcomputer (Appendix C).

### 6.4. Results.

Only one *X. maculatus* (red wag tail) female produced any fry, although in a sizeable number. A similar situation prevailed with *X. maculatus* (blue platy), where only one female produced three fry. An estimate of  $h^2$  could, therefore, not be obtained from these stocks.

The offspring produced and used in experiments for *X. maculatus* (yellow comet tail), *X. maculatus* (Vera Cruz) and *A. splendens* are shown in Tables 10, 11 and 12 respectively. The two *X. maculatus* stocks were infected at two separate points in time, whereas all *A. splendens* infections occurred simultaneously. In the case of *X. maculatus* (Vera Cruz) the two infections

occurred 9 days apart in late May, with 38 fish being infected on the first occasion and 51 fish infected on the second occasion. The two *X. maculatus* (yellow comet tail) infections occurred 18 days apart in late September (34 fish) and mid October (26 fish). *A. splendens* were infected in mid January (42 fish) only. For each stock of fish only a single parasite isolate was used, but these were not the same across stocks. *X. maculatus* (yellow comet tail) was infected with parasite isolate H, *X. maculatus* (Vera Cruz) with isolate P and *A. splendens* with isolate M (Appendix B). Mean ages at infection were  $44.8 \pm 1.2$  weeks for *X. maculatus* (yellow comet tail),  $58.0 \pm 3.3$  weeks for *X. maculatus* (Vera Cruz) and  $54.7 \pm 2.3$  weeks for *A. splendens*.

Whilst the numbers of broods are not ideal, (7 broods were infected for *X. maculatus*, yellow comet tail, 9 broods were infected for *X. maculatus*, Vera Cruz, and 8 broods were infected for *A. splendens*), sufficient data were obtained to enable an estimation of  $h^2$  to be made. The infection data<sup>are</sup> to be found in Tables 13, 14 and 15, with water quality parameters for these infections in Table 16.

For *A. splendens* nitrite and ammonia levels were monitored, twice daily, throughout the experiment. Those shown in Table 16 are for pre- and post-experiment only. When levels of nitrite exceeded 0.1 mg/l or ammonia exceeded 1.0 mg/l, partial water changes were performed, provided it was at least 18h after the initial infection. The temperatures used in the analysis were the mean temperatures of each tank throughout the experiment. These varied little : *X. maculatus* (yellow comet tail)  $21.8 \pm 0.2^\circ\text{C}$ , *X. maculatus* (Vera Cruz)  $25.2 \pm 0.3^\circ\text{C}$  and *A. splendens*  $24.8 \pm 0.4^\circ\text{C}$ .

The *X. maculatus* (Vera Cruz) were the smallest ( $2.29 \pm 0.79 \text{ cm}^2$ ) of the three stocks examined, with *X. maculatus* (yellow comet tail) and *A. splendens* being of similar size ( $6.11 \pm 1.40$  and  $5.68 \pm 1.59 \text{ cm}^2$  respectively). The male : female ratios of all offspring infected were 1 : 1.1 for *X. maculatus* (yellow comet tail), 1 : 1.3 for *X. maculatus* (Vera Cruz) and 1 : 1.8 for *A. splendens*. Parasite burdens averaged  $186.03 \pm 38.06$  and  $305.30 \pm 123.80$  trophozoites per fish on the

two occasions that *X. maculatus* (yellow comet tail) were infected. Such figures for *X. maculatus* (Vera Cruz) infections were  $7.11 \pm 6.34$  and  $124.76 \pm 61.02$  trophozoites per fish. Infection levels on *A. splendens* averaged  $16.90 \pm 6.52$  trophozoites per fish for all offspring infected. Mean infection levels for each of the broods and each of the sires are shown in Tables 13 to 15.

#### 6.4.1. Analysis of data.

The total counts of *I. multifiliis* on each fish were analysed initially using the Generalised Linear Interactive Modelling Package (GLIM) on a Prime computer. Factors included in this analysis were the surface area of the fish, the sex of the infected fish, the temperature of the water during infection and, except for *A. splendens* infections, the time of the experiment. Natural logarithmic transformed data was used to remove the correlations of means with variance found in untransformed data for each of the three stocks of fish (Figures 31 to 33).

The series of anova tables produced are shown in Tables 17 to 19. The time at which the experiment was performed was not applicable to *A. splendens*, but provided a significant amount of variation for both *X. maculatus* (yellow comet tail) (infections at 18 days apart,  $P < 0.001$ ) and *X. maculatus* (Vera Cruz) (infections at 9 days apart,  $P < 0.001$ ). The effect of variation due to temperature was not significant for any of the three stocks. As mentioned in the subsection above however, the variation in temperature was small (maximum standard deviation =  $0.42^{\circ}\text{C}$ ). The significance of variation due to the sex of the infected fish and variation due to the surface area of that fish varied between the three stocks of fish. Variation due to area did not form a significant amount of the variation in the case of *X. maculatus* (Vera Cruz) and *A. splendens* ( $P > 0.10$ ), but such variation was significant ( $P < 0.05$ ) for *X. maculatus* (yellow comet tail). It will be recalled that surface area values for the three species were  $2.29 \pm 0.79$ ,  $5.68 \pm 1.59$  and  $6.11 \pm 1.40$   $\text{cm}^2$  respectively. The relationship between surface area and infection levels for *X. maculatus* (yellow comet tail) was not a direct proportional one, but involved a factor of  $0.10 \pm 0.04$ . Hence, for *X. maculatus*

(yellow comet tail), increasing size of fish by 100% produced a 10% relative increase in parasite burden.

The sex of the infected fish had a significant effect in the case of *X. maculatus* (Vera Cruz) ( $P < 0.10$ ) and *A. splendens* ( $P < 0.05$ ), but such variation was not significant ( $P > 0.10$ ) for *X. maculatus* (yellow comet tail). Infection levels on males and females were  $226.1 \pm 108.7$  and  $247.9 \pm 100.7$  trophozoites per fish for *X. maculatus* (yellow comet tail),  $85.3 \pm 82.0$  and  $66.5 \pm 68.2$  trophozoites per fish for *X. maculatus* (Vera Cruz) and  $20.4 \pm 6.3$  and  $15.0 \pm 5.9$  trophozoites per fish for *A. splendens*. Hence, in both the significant cases the male fish harboured more parasites than female fish. It should also be mentioned that variation due to sex was the only significant factor for *A. splendens* (Table 19).

#### 6.4.2. Estimation of variance components and heritability.

The data, from each of the experiments here, falls into an unbalanced hierarchical design. With unequal numbers of dams per sire and progeny per dam the coefficients  $k_{1-3}$  are determined, as mentioned above, differently;

$$k_1 = \frac{n_{..} - \sum_i \frac{\sum_j n_{ij}^2}{n_i}}{n_d}$$

$$k_2 = \frac{\sum_i \frac{\sum_j n_{ij}^2}{n_i} - \frac{\sum_i \sum_j n_{ij}^2}{n_{..}}}{n_s}$$

$$k_3 = \frac{n_{..} - \frac{\sum_i n_i^2}{n_{..}}}{n_s}$$

where;

- $n_{..}$  = total number of progeny,
- $n_i$  = number of progeny per sire,
- $n_{ij}$  = number of progeny per dam,
- $n_s$  = degrees of freedom associated with sires,
- $n_d$  = degrees of freedom associated with dams.

Becker (1975) commented that the standard errors and confidence limits of heritability estimates have not been determined for unbalanced designs. Turner & Young (1969), though, do describe the computation of standard error for such heritability estimations. This was based upon the conservative approximation of Dickerson (1959). The approximate standard errors are;

1. For  $h_S^2$ ,

$$\sigma(h^2) \approx \frac{4A}{\sigma_T^2}$$

where,

$$A = \sqrt{\frac{2}{k_3} \left[ \frac{MS_s^2}{n_s} + \frac{MS_d^2}{n_d} \right]}$$

2. For  $h_D^2$ ,

$$\sigma(h^2) \approx \frac{4B}{\sigma_T^2}$$

where,

$$B = \sqrt{\frac{2}{k_1} \left[ \frac{MS_D^2}{n_d} + \frac{MS_O^2}{n_O} \right]}$$

3. For  $h_{S+D}^2$ ,

$$\sigma(h^2) \approx \frac{2\sqrt{A^2 + B^2 + 2C}}{\sigma_T^2}$$

where,

$$C = -\frac{k_2}{k_3} \left[ B^2 - \frac{2(MS_O)^2}{n_O k_1^2} \right]$$

and  $n_o$  is the degrees of freedom associated with the offspring.

Given the above computation a BASIC program was written (Appendix C) and the infection data was analysed on a BBC microcomputer. Three analyses were performed. The total count data and its natural logarithm were used in the analyses, but, since the factors of time of the experiment, surface area of the fish and sex of the infected fish had been shown to have varying significant effects on infection levels, another analysis was also performed. In this analysis

the fitted, or modelled, values of the GLIM analysis were used. These values, therefore, had effects due to time, surface area and sex removed, and provided a more accurate heritability estimation, since variation present will be due solely to differences between full and half-sib groups.

Anova tables are shown in Tables 20 to 22. These tables also show the percentage of the mean square accounted for by each level of the analysis of variance. It can be seen that in each case the distribution of mean squares is very different between the total count analysis and the modelled values analysis. The variance components and heritability values were estimated and are shown in Table 23. For *X. maculatus* (Vera Cruz) and *A. splendens* some values of  $\sigma_s^2$  and  $\sigma_D^2$  were computed which were less than zero. This, somewhat contradictory, situation of a negative variance component can be explained in terms of the significance of such a component. Tietjen & Moore (1968) describe a method of testing the significance of variance components, from an unbalanced design, by calculating the inverse of a matrix, whose elements include the coefficients of the variance components (here  $k_{1-3}$ ). The approximate  $F$ -test data is shown in Table 24. As can be seen, the degrees of freedom for the  $\sigma_s^2$   $F$ -ratios are low, which is the combined result of the low numbers of sires used and the poor breeding performance of those sires. The degrees of freedom for  $\sigma_D^2$  are greatly influenced by the numbers of females which produced offspring, which was also low here. Therefore due to the reasons above and the conservative nature of this  $F$  approximation, values were not significantly different from zero, at the 10% level, for all variance components except  $\sigma_D^2$ . Furthermore, none of the negative variance components were significantly different from zero, thus clarifying this apparent anomaly.

The effect of using logarithmic transformed data and the GLIM analysis upon heritability estimation can be clearly seen from Table 23. In each case, and especially with *A. splendens*, the heritability estimates differ, demonstrating that these factors can certainly mask the extent of additive variation in the population. The heritability values obtained using the modelled data are the

most precise estimates here with  $h_S^2$  being  $0.75 \pm 1.10$ ,  $0.23 \pm 1.04$  and  $0.00 \pm 1.05$  for *X. maculatus* (Vera Cruz), *X. maculatus* (yellow comet tail) and *A. splendens* respectively. Equivalent values of  $h_D^2$  were  $1.13 \pm 0.93$ ,  $0.92 \pm 1.06$  and  $1.54 \pm 1.40$ . Hence, it can be seen that  $h_D^2$  was greater than  $h_S^2$  for each stock of fish. Only in the case of  $h_S^2$  for *A. splendens* was a negative, and therefore zero, heritability value obtained. In the remaining cases, therefore, additive variance was found to be present, providing good potential for the improvement of resistance to *I. multifiliis* infection.

## 6.5. Discussion.

The only other study where heritability has been estimated for resistance to *I. multifiliis* is that of Bone (1983) where a red wagtail stock of *X. maculatus* was used. The value of  $\sigma_S^2$  was negative and effectively zero. This was assumed by Bone, but testing of  $\sigma_S^2$  by the above method of Tietjen & Moore (1968), showed that  $\sigma_S^2$  is not statistically different from zero,  $F=0.030_{[2,6,0]}$ ,  $P>0.10$ . Hence, this implies  $h_S^2=0.00 \pm 1.04$ . Bone (1983) did not proceed with a  $h_D^2$  calculation, but re-working of the data presents a figure of  $5.61 \pm 1.80$ . Again testing of  $\sigma_D^2$  was not performed, but such a calculation shows that  $\sigma_D^2$  is significant,  $F=146.75_{[6,124]}$ ,  $P<0.001$ . A natural logarithmic transformation of total parasite counts does not alter the significance of  $\sigma_S^2$  or  $\sigma_D^2$  and produces heritability estimations of  $0.00 \pm 1.01$  and  $5.28 \pm 1.75$  for  $h_S^2$  and  $h_D^2$  respectively. In Bone's case the between dams mean square accounted for 95% of the total variation. Here such values were much lower being 29.9% for *X. maculatus* (Vera Cruz), 33.8% for *X. maculatus* (yellow comet tail) and 42.9% for *A. splendens*, (based on the anova of GLIM modelled values).

In discussing the heritability values of Bone (1983) and those found here, one is presented with the problem of the significance of the variance components. It is not infrequently that heritability studies, based on an unbalanced design, are published (Tietjen & Moore, 1968). Commonly, the significance of variance components is not tested, since *F*-approximation tests are considered to be very conservative. An exception to this, is the body of work published on a

collection of Norwegian trout stocks that were investigated by a large group of workers (e.g. Gunnes & Gjedrem, 1978, 1981).

The maximum value of  $h^2$  is theoretically 1.0. The value of  $h_D^2$  for *X. maculatus* (Vera Cruz), however, was  $1.13 \pm 0.93$  and  $h_D^2$  for *A. splendens* was  $1.54 \pm 1.40$ . Heritability values greater than 1.0 have been reported elsewhere (see reviews by Gjedrem, 1983, and Tave, 1986). Durborow *et al* (1985) obtained a heritability of  $1.7 \pm 0.1$  for channel catfish fry mortality at 1.1 ppm  $O_2$ . In the majority of such cases, including the one cited, the estimation of heritability was by a method which also included  $V_D$  and/or  $V_M$ . From the division of each heritability estimation into the variance components involved (see page 112), it can be seen that  $h_D^2$  exceeds  $h_S^2$  by  $V_D + \frac{1}{2}V_{AA} + \frac{1}{2}V_{AD} + \frac{1}{4}V_{DD} + \frac{3}{8}V_{AAA} + 4V_M$ . Only two of these terms involve additive variance alone, and therefore the difference between  $h_D^2$  and  $h_S^2$  estimates is principally due to dominance ( $V_D$ ) and maternal ( $V_M$ ) variance. Differences in dam and sire heritability estimations have been recorded in several studies (Gunnes & Gjedrem, 1978 ; Refstie & Steine, 1978). Such differences were present here in each of the three stocks, but especially in the case of *A. splendens*, where only  $h_D^2$  is greater than zero. In addition, since it was always  $h_D^2$  which exceeded 1.0, it appears that non-additive genetic variation is the reason for such high heritability estimations and  $V_M$ .

It is difficult to accurately separate maternal and dominance components. Maternal effects are often only associated with young offspring. For example, Chevassus (1976) found a maternal effect in early growth of rainbow trout, but this effect disappeared after about 2 months. However, the offspring used here were quite mature (44.8 to 58.0 weeks - the typical life expectancy is approximately 102 weeks). Bones study (1983) of *X. maculatus* (red wagtail) used fish of of a similar age (46-54 weeks). Ayles (1974) calculated  $m$ , the magnitude of the sire variance relative to the dam variance component, as a function of the total variance. Calculation of such figures gives 0.10 for *X. maculatus* (Vera Cruz), 0.17 for *X. maculatus* (yellow comet tail), 0.34 for *A. splendens* and 0.88 for the *X. maculatus* (red wag tail) of Bone (1983).



Although the value of 0.88 for the *X. maculatus* (red wag tail) of Bone (1983) is much larger than the other figures it must be borne in mind that this is based on unmodelled data, and will therefore include the confounding effects of size, time and sex.

All of these species are livebearing fish, which actively nourish their young in development. In xiphophorid fish there is no change in dry weight of embryos during gestation (Wourms, 1981). Scrimshaw (1945) estimated that this is due to a 30-40% contribution from the mother, to maintain the weight of the developing embryo. However, the nourishment of developing *A. splendens* fry is even greater. During development in the mother an *A. splendens* embryo increases some 8,430% in dry weight (Wourms, 1981). This is achieved via the nutritive trophotaeniae (Turner, 1937), which is almost equivalent to the mammalian placenta in function. This then is one possible source of maternal variation, by protection conferred on the offspring, by the mother, through the nourishment which takes place. In this study, the maternal effects of *A. splendens* were approximately three or four times those found in the *X. maculatus* stocks.

The standard errors of the heritability estimations appear to be quite substantial (0.93 - 1.40). This is undoubtedly due to the fact that the conservative approximation of Dickerson (1959) had to be used because of the unbalanced nature of the data and the limited number of dams and sires. Standard errors in the literature are also often quite high. Reagan (1979) obtained heritabilities of  $0.92 \pm 1.08$  for 30-day weight and  $1.22 \pm 1.11$  for 30-day length in channel catfish. Smoker (1981) obtained heritabilities for the number of days between spawning and emergence in two strains of chum salmon : Kilches river  $0.0 \pm 1.1$  and Whiskey river  $0.8 \pm 1.1$ . However, the heritabilities here include the highest yet recorded for disease resistance in fish. McIntyre & Amend (1978) observed the resistance to IHN virus in sockeye salmon. Heritability values of  $0.27 \pm 2.6$  to  $0.38 \pm 5.1$  were obtained. Smoker (1981) determined the heritability for tolerance to *Vibrio anguillarum* in chum salmon as  $0.5 \pm 0.6$ . The heritability for the same trait in atlantic salmon has been estimated

as  $0.11 \pm 0.06$  (Gjedrem & Aulstad, 1974). The value of  $h_s^2$  for *X. maculatus* (Vera Cruz) here was  $0.75 \pm 1.10$  and therefore exceeds the heritability values of other fish disease studies and has a similar standard error to other recorded heritability estimates. Tave (1986) stated that when  $h^2 > 0.25$  exploitation of the population via selection methods is quite effective at producing change. There are, then, good possibilities for the selection for increased resistance to *I. multifiliis* infection. The presence of a zero  $h_s^2$  for *A. splendens* though stresses the need to determine heritability values for commercially farmed species.

Table 10 *Xiphophorus maculatus* (yellow comet tail) fry produced and infected in the heritability half-sib analysis.

|          | Dam. | Age at infection (weeks). | Number infected. | Date of birth. |
|----------|------|---------------------------|------------------|----------------|
| Sire I   | A    | 45.7                      | 1                | 22/11/85       |
|          | B    | 46.0                      | 1                | 20/11/85       |
|          | C    | -----                     | 0                | -----          |
|          | D    | -----                     | 0                | -----          |
| Sire II  | A    | 45.9                      | 8                | 21/11/85       |
|          | B    | -----                     | 0                | -----          |
|          | C    | -----                     | 0                | -----          |
|          | D    | -----                     | 0                | -----          |
| Sire III | A    | 43.5 & 45.8               | 20               | 21-22/11/85    |
|          | B    | -----                     | 0                | -----          |
|          | C    | -----                     | 0                | -----          |
|          | D    | -----                     | 0                | -----          |
| Sire IV  | A    | 43.7                      | 9                | 20/11/85       |
|          | B    | 43.3 & 45.6               | 16               | 22-24/11/85    |
|          | C    | 43.6                      | 5                | 21/11/85       |
|          | D    | -----                     | 0                | -----          |
| Sire V   | A    | -----                     | 0                | -----          |
|          | B    | -----                     | 0                | -----          |
|          | C    | -----                     | 0                | -----          |
|          | D    | -----                     | 0                | -----          |
| Sire VI  | A    | -----                     | 0                | -----          |
|          | B    | -----                     | 0                | -----          |
|          | C    | -----                     | 0                | -----          |
|          | D    | -----                     | 0                | -----          |

Table 11 *Xiphophorus maculatus* (Vera Cruz) fry produced and infected in the heritability half-sib analysis.

|          | Dam. | Age at infection (weeks). | Number infected. | Date of birth. |
|----------|------|---------------------------|------------------|----------------|
| Sire I   | A    | 63.4                      | 6                | 18-21/2/87     |
|          | B    | 56.4                      | 3                | 9/4/87         |
|          | C    | -----                     | 0                | 13-15/4/87     |
|          | D    | -----                     | 0                | -----          |
| Sire II  | A    | 57.0 & 64.1               | 21               | 14/2/87        |
|          | B    | 54.6 & 55.9               | 19               | 22/4/87        |
|          | C    | 54.0                      | 4                | 24-27/4/87     |
|          | D    | -----                     | 0                | 30-31/3/87     |
| Sire III | A    | 61.0                      | 10               | 23-25/3/87     |
|          | B    | 59.0                      | 6                | 1-4/4/87       |
|          | C    | -----                     | 0                | 17/3/87        |
|          | D    | -----                     | 0                | 12/4/87        |
| Sire IV  | A    | 56.6 & 58.0               | 9                | 8/4/87         |
|          | B    | 56.0                      | 11               | 21/4/87        |
|          | C    | -----                     | 0                | 27-29/4/87     |
|          | D    | -----                     | 0                | -----          |
| Sire V   | A    | -----                     | 0                | -----          |
|          | B    | -----                     | 0                | -----          |
|          | C    | -----                     | 0                | -----          |
|          | D    | -----                     | 0                | -----          |
| Sire VI  | A    | -----                     | 0                | -----          |
|          | B    | -----                     | 0                | -----          |
|          | C    | -----                     | 0                | -----          |
|          | D    | -----                     | 0                | -----          |

Table 12 *Ameca splendens* fry produced and infected in the heritability half-sib analysis.

|          | Dam. | Age at infection (weeks). | Number infected. | Date of birth. |
|----------|------|---------------------------|------------------|----------------|
| Sire I   | A    | 56.9                      | 9                | 11/12/86       |
|          | B    | -----                     | 0                | 21/10/86       |
|          | C    | -----                     | 0                | -----          |
|          | D    | -----                     | 0                | -----          |
| Sire II  | A    | 51.3                      | 8                | 19/01/87       |
|          | B    | -----                     | 0                | 23/09/86       |
|          | C    | -----                     | 0                | -----          |
|          | D    | -----                     | 0                | -----          |
| Sire III | A    | 55.9                      | 6                | 10/11/86       |
|          | B    | 58.0                      | 6                | 03/12/86       |
|          | C    | -----                     | 0                | 26/02/87       |
|          | D    | -----                     | 0                | -----          |
| Sire IV  | A    | 54.1                      | 2                | 31/12/86       |
|          | B    | 51.7                      | 8                | 16/01/87       |
|          | C    | -----                     | 0                | 05/01/87       |
|          | D    | -----                     | 0                | -----          |
| Sire V   | A    | 55.3                      | 1                | 22/12/86       |
|          | B    | 54.3                      | 2                | 30/12/86       |
|          | C    | -----                     | 0                | 13/11/86       |
|          | D    | -----                     | 0                | -----          |
| Sire VI  | A    | -----                     | 0                | 23/09/86       |
|          | B    | -----                     | 0                | -----          |
|          | C    | -----                     | 0                | -----          |
|          | D    | -----                     | 0                | -----          |

Table 13 Infection levels of *Ichthyophthirius multifiliis* on *Xiphophorus maculatus* (yellow comet tail platy) used in the heritability estimation.

| Sire     | Dam (infection date). | Trophozoites per fish. |     |                     |     |                     |     |                     |     |               |               | Mean number of trophozoites per fish. |  |  |
|----------|-----------------------|------------------------|-----|---------------------|-----|---------------------|-----|---------------------|-----|---------------|---------------|---------------------------------------|--|--|
|          |                       | A (t <sub>1</sub> )    |     | B (t <sub>1</sub> ) |     | A (t <sub>2</sub> ) |     | B (t <sub>2</sub> ) |     | Tank          | Dam           | Sire                                  |  |  |
| Sire I   | A (t <sub>1</sub> )   | 597                    |     |                     |     |                     |     |                     |     | 597           | 597           | 501.0 ± 135.8                         |  |  |
|          | B (t <sub>1</sub> )   | 405                    |     |                     |     |                     |     |                     | 405 |               |               |                                       |  |  |
| Sire II  | A (t <sub>1</sub> )   | 235                    | 195 | 455                 | 404 | 239                 | 372 | 200                 | 217 | 289.6 ± 103.5 | 289.6 ± 103.5 | 289.6 ± 36.6                          |  |  |
|          | A (t <sub>2</sub> )   | 223                    | 197 | 201                 | 137 | 244                 | 171 | 183                 | 163 | 190.6 ± 31.4  | 207.0 ± 50.2  | 207.0 ± 50.2                          |  |  |
| Sire III | A (t <sub>1</sub> )   | 235                    | 207 | 347                 | 186 | 191                 | 288 | 219                 | 244 | 223.4 ± 61.2  |               |                                       |  |  |
|          | A (t <sub>2</sub> )   | 206                    | 173 | 175                 | 167 | 233                 | 247 | 219                 | 179 | 197.8 ± 29.4  | 197.8 ± 29.4  | 226.8 ± 104.5                         |  |  |
| Sire IV  | B (t <sub>2</sub> )   | 226                    | 279 | 147                 | 190 | 162                 | 172 | 226                 | 195 | 188.2 ± 45.0  | 266.7 ± 127.0 |                                       |  |  |
|          | B (t <sub>1</sub> )   | 462                    | 507 | 337                 | 507 | 250                 | 322 |                     |     | 397.5 ± 108.9 |               |                                       |  |  |
|          | C (t <sub>2</sub> )   | 157                    | 168 | 188                 | 160 | 84                  |     |                     |     | 151.4 ± 39.6  | 151.4 ± 39.6  |                                       |  |  |

Table 14 Infection levels of *Ichthyophthirius multifiliis* on *Xiphophorus maculatus* (Vera Cruz) used in the heritability estimation.

|          | Dam (infection date) | Trophozoites per fish. |     |     |     |     |     |     |     |     |     | Mean number of trophozoites per fish. |     |              |              |              |
|----------|----------------------|------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|---------------------------------------|-----|--------------|--------------|--------------|
|          |                      | 3                      | 22  | 6   | 2   | 0   | 3   | 1   | 6   | 2   | 2   | 225                                   | 146 | 163          | Tank         | Dam          |
| Sire I   | A (t <sub>1</sub> )  | 11                     | 2   | 10  |     |     |     |     |     |     |     |                                       |     | 6.0 ± 8.1    | 6.0 ± 8.1    | 6.6 ± 6.9    |
|          | B (t <sub>1</sub> )  |                        |     |     |     |     |     |     |     |     |     |                                       |     | 7.7 ± 4.9    | 7.7 ± 4.9    |              |
| Sire II  | A (t <sub>1</sub> )  | 11                     | 6   | 9   | 20  | 24  | 1   | 1   | 6   | 2   |     |                                       |     | 8.9 ± 8.2    | 109.6 ± 95.8 | 76.7 ± 80.7  |
|          | A (t <sub>2</sub> )  | 211                    | 195 | 241 | 164 | 246 | 120 | 141 | 133 | 237 | 225 | 146                                   | 163 | 185.2 ± 46.0 |              |              |
|          | B (t <sub>1</sub> )  | 12                     | 4   | 7   | 5   | 2   | 4   | 9   | 6   | 4   |     |                                       |     | 5.9 ± 3.1    | 53.4 ± 51.3  |              |
|          | B (t <sub>2</sub> )  | 92                     | 86  | 92  | 80  | 68  | 116 | 85  | 76  | 89  | 41  | 177                                   |     | 96.1 ± 31.1  |              |              |
|          | C (t <sub>1</sub> )  | 11                     | 19  | 9   | 18  |     |     |     |     |     |     |                                       |     | 14.2 ± 5.0   | 14.2 ± 5.0   |              |
| Sire III | A (t <sub>1</sub> )  | 108                    | 74  | 71  | 49  | 128 | 37  | 124 | 43  | 48  |     |                                       |     | 72.3 ± 35.4  | 72.3 ± 35.4  | 74.2 ± 30.3  |
|          | B (t <sub>1</sub> )  | 89                     | 86  | 110 | 70  | 59  | 50  |     |     |     |     |                                       |     | 77.3 ± 22.0  | 77.3 ± 22.0  |              |
| Sire IV  | A (t <sub>1</sub> )  | 7                      | 1   | 0   | 2   | 2   | 2   | 7   |     |     |     |                                       |     | 3.0 ± 2.8    | 16.7 ± 29.5  | 100.7 ± 85.8 |
|          | B (t <sub>1</sub> )  | 87                     | 42  |     |     |     |     |     |     |     |     |                                       |     | 64.5 ± 31.8  |              |              |
|          |                      | 137                    | 203 | 191 | 181 | 201 | 186 | 98  | 232 | 164 | 105 | 166                                   |     | 169.5 ± 41.5 | 169.5 ± 41.5 |              |

Table 15 Infection levels of *Icthyophthirius multifiliis* on *Ameba splendens* used in the heritability estimation.

|          | Dam | Trophozoites per fish |    |    |    |    |    |    |    |    |              | Mean number of trophozoites per fish |  |
|----------|-----|-----------------------|----|----|----|----|----|----|----|----|--------------|--------------------------------------|--|
|          |     | 9                     | 17 | 8  | 30 | 23 | 25 | 15 | 9  | 9  | Dam          | Sire                                 |  |
| Sire I   | A   | 9                     | 17 | 8  | 30 | 23 | 25 | 15 | 9  | 9  | 16.11 ± 8.21 | 16.11 ± 8.21                         |  |
| Sire II  | A   | 23                    | 21 | 27 | 21 | 11 | 15 | 25 | 21 | 21 | 20.50 ± 5.21 | 20.50 ± 5.21                         |  |
| Sire III | A   | 7                     | 22 | 20 | 9  | 15 | 12 |    |    |    | 14.17 ± 5.98 | 16.50 ± 7.33                         |  |
|          | B   | 30                    | 18 | 17 | 13 | 27 | 8  |    |    |    | 18.83 ± 8.33 |                                      |  |
| Sire IV  | A   | 10                    | 19 |    |    |    |    |    |    |    | 14.50 ± 6.36 | 16.70 ± 4.88                         |  |
|          | B   | 24                    | 18 | 11 | 16 | 22 | 21 | 14 | 12 |    | 17.25 ± 4.80 |                                      |  |
| Sire V   | A   | 16                    |    |    |    |    |    |    |    |    | 16.00        | 12.00 ± 3.60                         |  |
|          | B   | 9                     | 11 |    |    |    |    |    |    |    | 10.00 ± 1.41 |                                      |  |



Table 16 Water quality parameters pre- and post-experiment for the heritability half-sib analysis.<sup>7</sup>

|   | Temperature (°C) | pH        | Nitrate (mg/l) | Nitrite (mg/l) | Ammonia (mg/l) | Hardness (°d) |
|---|------------------|-----------|----------------|----------------|----------------|---------------|
| <i>Xiphophorus maculatus</i><br>(yellow comet tail) |                  |           |                |                |                |               |
| Pre-experiment                                      | 21.7 ± 0.4       | 7.5 ± 0.2 | <5             | -----          | <0.3           | 3.1 ± 0.3     |
| Post-experiment                                     | 21.8 ± 0.3       | 7.4 ± 0.2 | <5             | -----          | 0              | 3.4 ± 0.7     |
| <i>Xiphophorus maculatus</i><br>(Vera cruz)         |                  |           |                |                |                |               |
| Pre-experiment                                      | 25.0 ± 0.2       | 7.4 ± 0.1 | 5.5 ± 1.4      | 0.01 ± 0.01    | 0.09 ± 0.05    | 0 - 5         |
| Post-experiment                                     | 25.5 ± 0.6       | 7.3 ± 0.2 | 5.2 ± 1.7      | 0.01 ± 0.01    | 0.36 ± 0.08    | -----         |
| <i>Ameca splendens</i>                              |                  |           |                |                |                |               |
| Pre-experiment                                      | 25.8 ± 0.3       | 7.0 ± 0.5 | 3.2 ± 0.5      | 0.01 ± 0.01*   | 0.97 ± 0.80*   | 0 - 5         |
| Post-experiment                                     | 24.1 ± 0.5       | 6.9 ± 0.7 | 6.0 ± 3.2      | 0.06 ± 0.09*   | 1.0 ± 1.1*     | 5 - 10        |

<sup>7</sup>An asterisk indicates where parameters were monitored twice daily throughout the experiment. Partial water changes were performed when nitrite exceeded 0.1 mg/l or ammonia exceeded 1.0 mg/l.

Figure 31 Plots of residual against fitted values after the GLIM analysis for total *Ichthyophthirius multifiliis* burden on *Xiphophorus maculatus* (yellow comet tail) a. Untransformed data, b. Natural logarithmic transformed data.

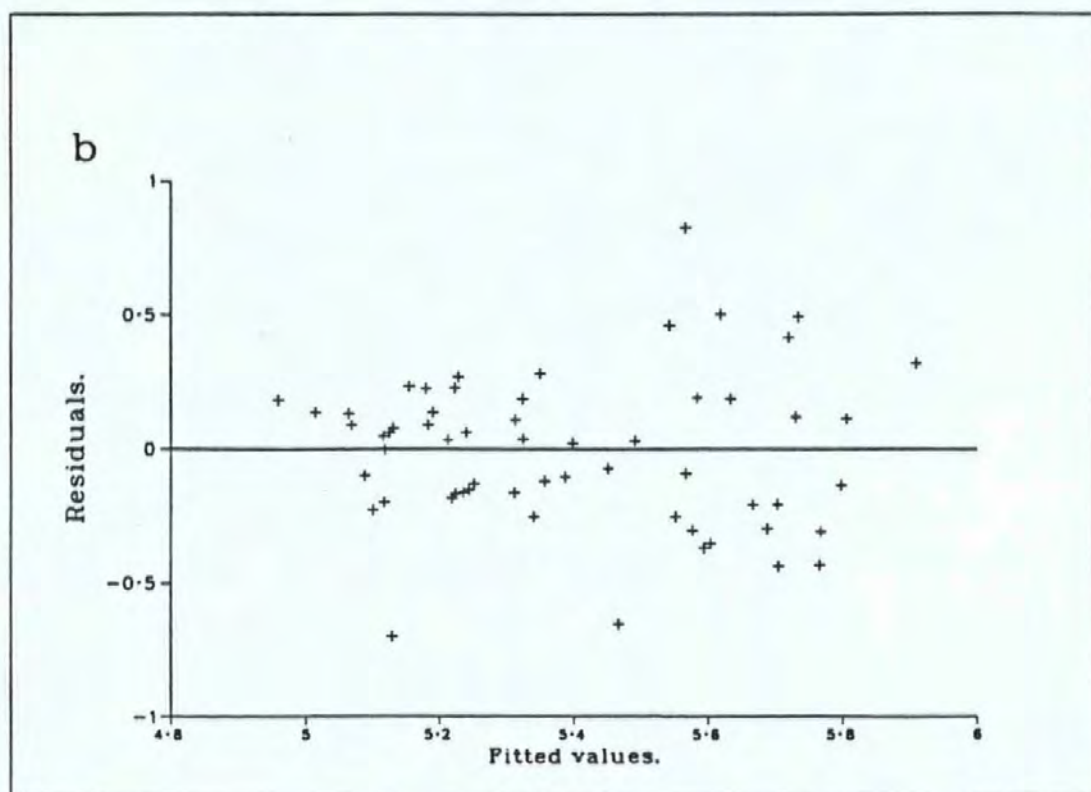
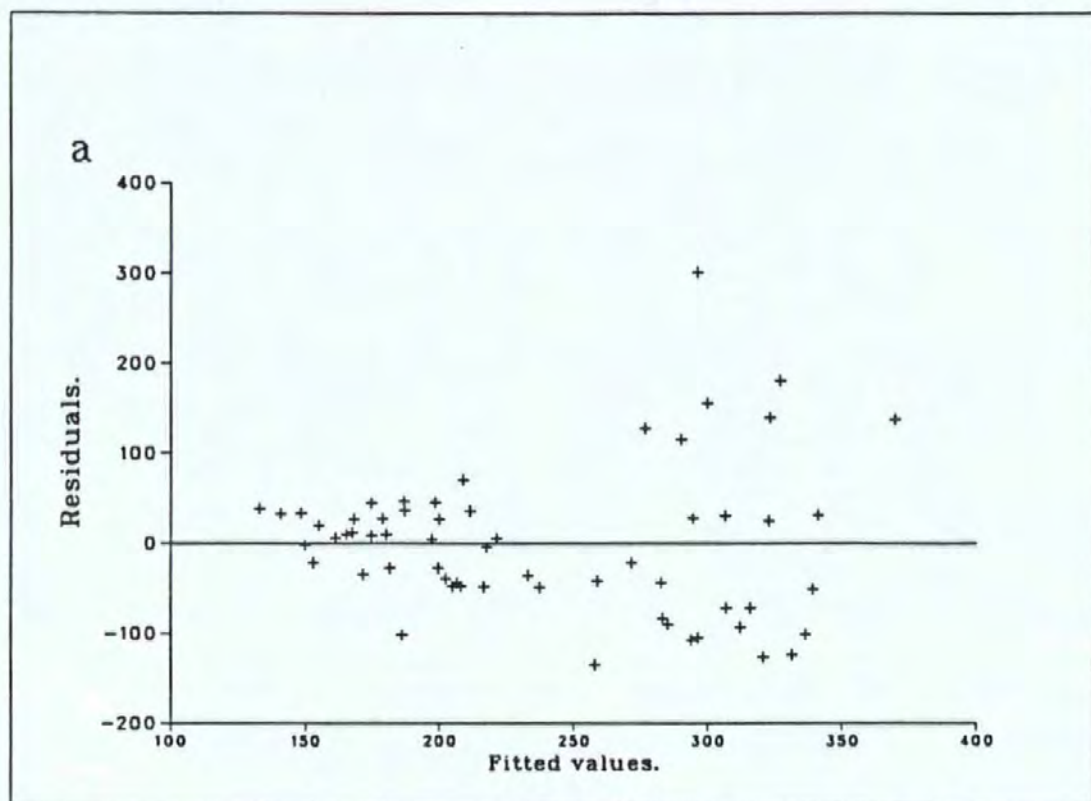


Figure 32 Plots of residual against fitted values after the GLIM analysis for total *Ichthyophthirius multifiliis* burden on *Xiphophorus maculatus* (Vera Cruz) a. Untransformed data, b. Natural logarithmic transformed data.

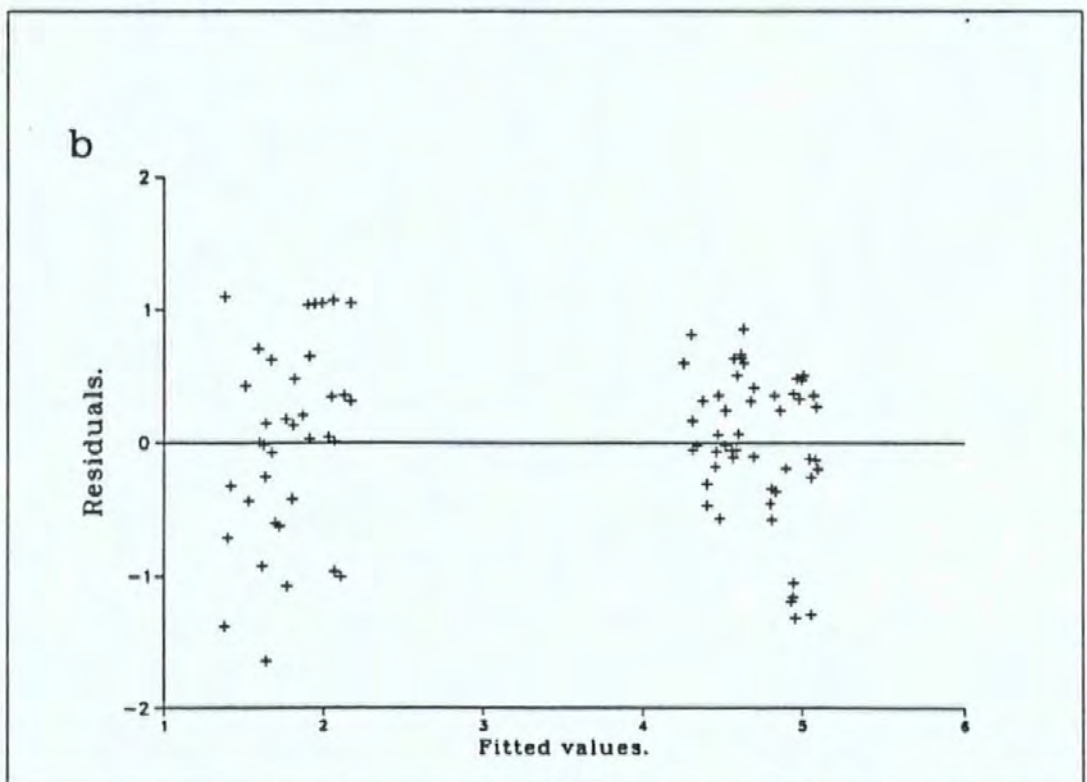
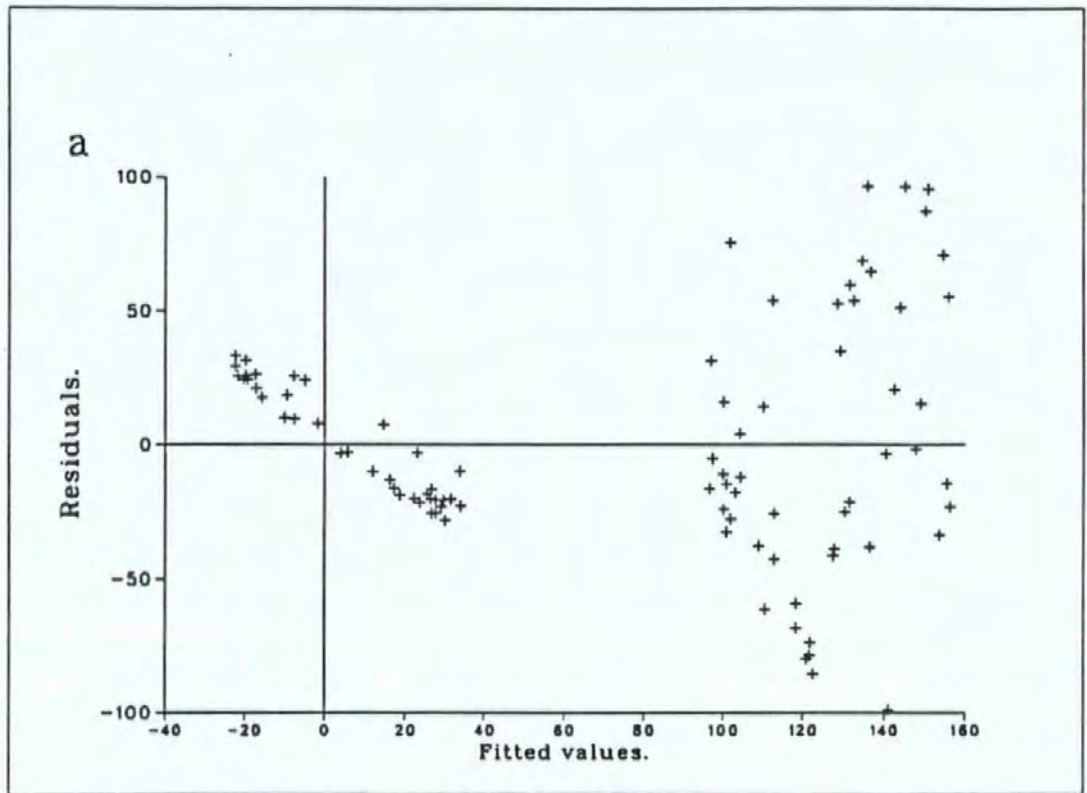
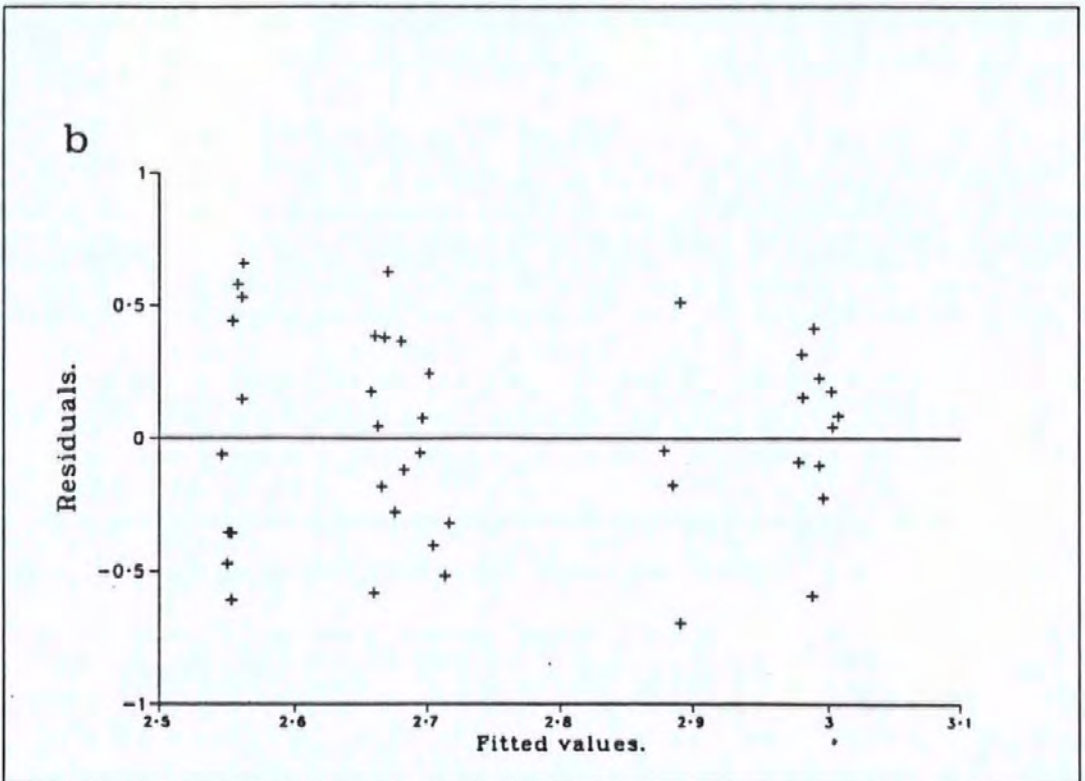
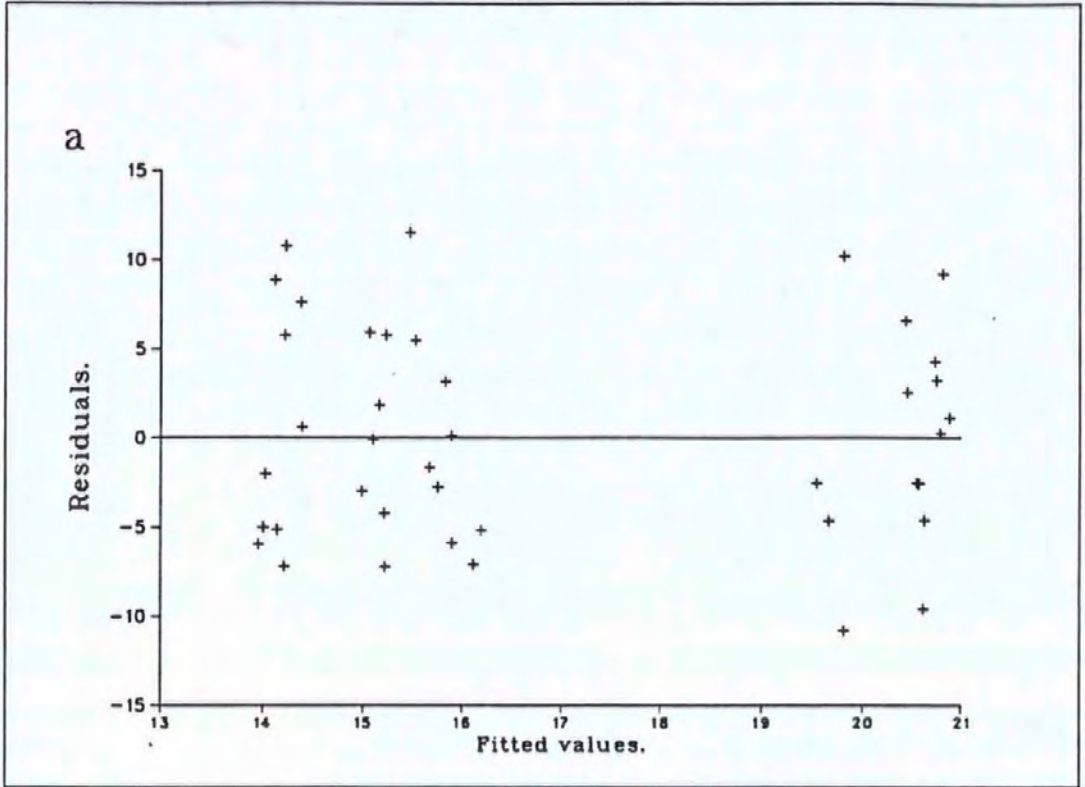


Figure 33 Plots of residual against fitted values after the GLIM analysis for total *Ichthyophthirius multifiliis* burden on *Ameioba splendens* a. Untransformed data, b. Natural logarithmic transformed data.



**Table 17** Analysis of variance tables for each of the variables in the heritability determination infections of *Xiphophorus maculatus* (yellow comet tail). Natural log transformed data.<sup>8</sup>

| Source of variation  | d.f. | Sum of Squares | Mean Squares | F      | P    |
|--|------|----------------|--------------|--------|------|
| Effects due to time of the experiment, sex and temperature.  | 3    | 3.050          | 1.017        | 11.640 | **** |
| Effects due to area.   | 1    | 0.546          | 0.546        | 6.252  | **   |
| Residual.  | 55   | 4.803          | 0.087        |        |      |
| Total  | 59   | 8.399          |              |        |      |
| Effects due to area, sex and temperature.                    | 3    | 1.268          | 0.423        | 4.840  | ***  |
| Effects due to time of of the experiment.                    | 1    | 2.328          | 2.328        | 26.658 | **** |
| Residual.  | 55   | 4.803          | 0.087        |        |      |
| Total  | 59   | 8.399          |              |        |      |
| Effects due to area, time of the experiment and temperature. | 3    | 3.592          | 1.197        | 13.711 | **** |
| Effects due to sex.  | 1    | 0.004          | 0.004        | 0.046  | NS   |
| Residual.  | 55   | 4.803          | 0.087        |        |      |
| Total  | 59   | 8.399          |              |        |      |
| Effects due to area, time of the experiment and sex.         | 3    | 3.447          | 1.149        | 13.157 | **** |
| Effects due to temperature.                                  | 1    | 0.149          | 0.149        | 1.706  | NS   |
| Residual.  | 55   | 4.803          | 0.087        |        |      |
| Total  | 59   | 8.399          |              |        |      |

<sup>8</sup>Significance levels as in Table 4.

**Table 18** Analysis of variance tables for each of the variables in the heritability determination infections of *Xiphophorus maculatus* (Vera Cruz). Natural log (y+1) transformed data.<sup>9</sup>

| Source of variation  | d.f. | Sum of Squares | Mean Squares | F       | P    |
|--|------|----------------|--------------|---------|------|
| Effects due to time of the experiment, sex and temperature.  | 3    | 190.140        | 63.380       | 155.761 | **** |
| Effects due to area.   | 1    | 0.980          | 0.980        | 2.408   | NS   |
| Residual.  | 84   | 34.180         | 0.407        |         |      |
| Total  | 88   | 225.300        |              |         |      |
| Effects due to area, sex and temperature.                    | 3    | 157.150        | 52.383       | 128.736 | **** |
| Effects due to time of of the experiment.                    | 1    | 33.970         | 33.970       | 83.484  | **** |
| Residual.  | 84   | 34.180         | 0.407        |         |      |
| Total  | 88   | 225.300        |              |         |      |
| Effects due to area, time of the experiment and temperature. | 3    | 189.970        | 63.323       | 155.622 | **** |
| Effects due to sex.  | 1    | 1.150          | 1.150        | 2.826   | *    |
| Residual.  | 84   | 34.180         | 0.407        |         |      |
| Total  | 88   | 225.300        |              |         |      |
| Effects due to area, time of the experiment and sex.         | 3    | 190.540        | 63.513       | 156.090 | **** |
| Effects due to temperature.                                  | 1    | 0.580          | 0.580        | 1.425   | NS   |
| Residual.  | 84   | 34.180         | 0.407        |         |      |
| Total  | 88   | 225.300        |              |         |      |

<sup>9</sup>Significance levels as in Table 4.

**Table 19** Analysis of variance tables for each of the variables in the heritability determination infections of *Ameba splendens*. Natural log transformed data.<sup>10</sup>

| Source of variation                  | d.f. | Sum of Squares | Mean Squares | F     | P  |
|--------------------------------------|------|----------------|--------------|-------|----|
| Effects due to sex and temperature.  | 2    | 1.214          | 0.607        | 4.037 | ** |
| Effects due to area.                 | 1    | 0.006          | 0.006        | 0.040 | NS |
| Residual.                            | 38   | 5.714          | 0.1504       |       |    |
| Total                                | 41   | 6.934          |              |       |    |
| Effects due to area and temperature. | 2    | 0.298          | 0.149        | 0.991 | NS |
| Effects due to sex.                  | 1    | 0.922          | 0.922        | 6.131 | ** |
| Residual.                            | 38   | 5.714          | 0.1504       |       |    |
| Total                                | 41   | 6.934          |              |       |    |
| Effects due to area and sex.         | 2    | 1.093          | 0.546        | 3.634 | ** |
| Effects due to temperature.          | 1    | 0.127          | 0.127        | 0.845 | NS |
| Residual.                            | 38   | 5.714          | 0.1504       |       |    |
| Total                                | 41   | 6.934          |              |       |    |

<sup>10</sup>Significance levels as in Table 4.

Table 20 Analysis of variance tables for each of the three heritability estimations for *Xiphophorus maculatus* (yellow comet tail).

| Source of variation.            | d.f. | Sum of squares. | Mean Squares. | % of analysis mean square total. |
|---------------------------------|------|-----------------|---------------|----------------------------------|
| <b>Total count analysis</b>     |      |                 |               |                                  |
| Between sires                   | 3    | 182673          | 60879         | 64.3                             |
| Between dams within sires       | 3    | 79895           | 26631         | 28.1                             |
| Between progeny within dams     | 53   | 377928          | 7131          | 7.5                              |
| <b>Ln(Total count) analysis</b> |      |                 |               |                                  |
| Between sires                   | 3    | 1.9217          | 0.6406        | 57.7                             |
| Between dams within sires       | 3    | 1.1015          | 0.3672        | 33.1                             |
| Between progeny within dams     | 53   | 5.3761          | 0.1014        | 9.1                              |
| <b>Modelled values analysis</b> |      |                 |               |                                  |
| Between sires                   | 3    | 0.6944          | 0.2315        | 55.1                             |
| Between dams within sires       | 3    | 0.4261          | 0.1420        | 33.8                             |
| Between progeny within dams     | 53   | 2.4762          | 0.0467        | 11.1                             |



Table 21 Analysis of variance tables for each of the three heritability estimations for *Xiphophorus maculatus* (Vera Cruz).

| Source of variation.            | d.f. | Sum of squares. | Mean Squares. | % of analysis mean square total. |
|---------------------------------|------|-----------------|---------------|----------------------------------|
| <b>Total count analysis</b>     |      |                 |               |                                  |
| Between sires                   | 3    | 55483           | 18494         | 33.8                             |
| Between dams within sires       | 5    | 164354          | 32871         | 60.1                             |
| Between progeny within dams     | 80   | 269267          | 3366          | 6.1                              |
| <b>Ln(Total count) analysis</b> |      |                 |               |                                  |
| Between sires                   | 3    | 44.92           | 14.97         | 49.4                             |
| Between dams within sires       | 5    | 67.28           | 13.46         | 44.4                             |
| Between progeny within dams     | 80   | 149.53          | 1.87          | 6.2                              |
| <b>Modelled values analysis</b> |      |                 |               |                                  |
| Between sires                   | 3    | 50.27           | 16.76         | 65.1                             |
| Between dams within sires       | 5    | 38.50           | 7.70          | 29.9                             |
| Between progeny within dams     | 80   | 102.49          | 1.28          | 5.0                              |

Table 22 Analysis of variance tables for each of the three heritability estimations for *Ameca splendens*.

| Source of variation.            | d.f. | Sum of squares. | Mean Squares. | % of analysis mean square total. |
|---------------------------------|------|-----------------|---------------|----------------------------------|
| <b>Total count analysis</b>     |      |                 |               |                                  |
| Between sires                   | 4    | 183.63          | 45.91         | 37.4                             |
| Between dams within sires       | 3    | 101.43          | 33.81         | 28.4                             |
| Between progeny within dams     | 34   | 1458.56         | 42.90         | 35.0                             |
| <b>Ln(Total count) analysis</b> |      |                 |               |                                  |
| Between sires                   | 4    | 0.8017          | 0.2004        | 39.1                             |
| Between dams within sires       | 3    | 0.4322          | 0.1441        | 28.1                             |
| Between progeny within dams     | 34   | 5.7005          | 0.1677        | 32.7                             |
| <b>Modelled values analysis</b> |      |                 |               |                                  |
| Between sires                   | 4    | 0.2506          | 0.0626        | 41.8                             |
| Between dams within sires       | 3    | 0.1931          | 0.0644        | 42.9                             |
| Between progeny within dams     | 34   | 0.7767          | 0.0228        | 15.2                             |

Table 23 Sire and dam variance components and heritability estimations for *Xiphophorus maculatus* (yellow comet tail platy), *X. maculatus* (Vera Cruz) and *Ameca splendens*.<sup>11</sup>

| Component.                               | Total count analysis. | Ln(Total count) analysis. | Modelled values analysis. |
|--|-----------------------|---------------------------|---------------------------|
| <i>X. maculatus</i><br>yellow comet tail |                       |                           |                           |
| $\sigma_s^2$                             | 2069.72               | 0.0126                    | 0.0038                    |
| $\sigma_D^2$                             | 3089.92**             | 0.0421**                  | 0.0151**                  |
| $h_s^2$                                  | 0.67 ± 1.19           | 0.32 ± 1.12               | 0.23 ± 1.04               |
| $h_D^2$                                  | 1.01 ± 1.06           | 1.08 ± 1.10               | 0.92 ± 1.06               |
| $h_{s+D}^2$                              | 0.84 ± 0.77           | 0.70 ± 0.68               | 0.58 ± 0.62               |
| <i>X. maculatus</i><br>Vera cruz         |                       |                           |                           |
| $\sigma_s^2$                             | -764.08               | 0.0644                    | 0.4536                    |
| $\sigma_D^2$                             | 3151.62****           | 1.2377****                | 0.6857****                |
| $h_s^2$                                  | -0.53 ± 0.95          | 0.08 ± 0.98               | 0.75 ± 1.10               |
| $h_D^2$                                  | 2.19 ± 1.24           | 1.56 ± 1.07               | 1.13 ± 0.93               |
| $h_{s+D}^2$                              | 0.83 ± 0.69           | 0.82 ± 0.70               | 0.94 ± 0.78               |
| <i>A. splendens</i>                      |                       |                           |                           |
| $\sigma_s^2$                             | 2.32                  | 0.0091                    | -0.0040                   |
| $\sigma_D^2$                             | -2.59                 | -0.0067                   | 0.0118*                   |
| $h_s^2$                                  | 0.22 ± 0.70           | 0.21 ± 0.73               | -0.53 ± 1.05              |
| $h_D^2$                                  | -0.24 ± 0.89          | -0.16 ± 0.91              | 1.54 ± 1.40               |
| $h_{s+D}^2$                              | -0.01 ± 0.32          | 0.03 ± 0.32               | 0.51                      |

<sup>11</sup>Significance levels as in Table 4.

Table 24 Significance of sire and dam variance components using the approximate  $F$ -test of Tietjen & Moore (1968).<sup>12</sup>

|   | Analysis.                           | Null hypothesis. | Approximate $F$ -ratio.                 |   |
|---|-------------------------------------|------------------|---|---|
| <i>Xiphophorus maculatus</i> ,<br>yellow comet platy. | Total count.                        | $\sigma_S^2 = 0$ | 1.729 <sub>[3,2,5]</sub>                |   |
|   |                                     | $\sigma_D^2 = 0$ | 3.735 <sup>**</sup> <sub>[3,53]</sub>   |   |
|   |                                     | $\sigma_B^2 = 0$ | 1.323 <sub>[3,2,5]</sub>                |   |
|   | Ln(Total count).                    | $\sigma_S^2 = 0$ | 3.620 <sup>**</sup> <sub>[3,53]</sub>   |   |
|   |                                     | $\sigma_D^2 = 0$ | 1.258 <sub>[3,2,4]</sub>                |   |
|   | Modelled values.                    | $\sigma_S^2 = 0$ | 3.040 <sup>**</sup> <sub>[3,53]</sub>   |   |
|   |                                     |                  |   |   |
|   | <i>X. maculatus</i> ,<br>Vera cruz. | Total count.     | $\sigma_S^2 = 0$                        | 0.552 <sub>[3,5]</sub>                  |
|   |                                     |                  | $\sigma_D^2 = 0$                        | 9.776 <sup>****</sup> <sub>[5,80]</sub> |
| $\sigma_B^2 = 0$                                      |                                     |                  | 1.092 <sub>[3,5]</sub>                  |   |
| Ln(Total count).                                      |                                     | $\sigma_S^2 = 0$ | 7.199 <sup>****</sup> <sub>[5,80]</sub> |   |
|   |                                     | $\sigma_D^2 = 0$ | 2.137 <sub>[3,5]</sub>                  |   |
| Modelled values.                                      |                                     | $\sigma_S^2 = 0$ | 6.011 <sup>****</sup> <sub>[5,80]</sub> |   |
|   |                                     |                  |   |   |
| <i>Ameca splendens</i> .                              |                                     | Total count.     | $\sigma_S^2 = 0$                        | 1.700 <sub>[4,0,6]</sub>                |
|   |                                     |                  | $\sigma_D^2 = 0$                        | 0.788 <sub>[3,34]</sub>                 |
|   | $\sigma_B^2 = 0$                    |                  | 1.586 <sub>[4,0,7]</sub>                |   |
|   | Ln(Total count).                    | $\sigma_S^2 = 0$ | 0.859 <sub>[3,34]</sub>                 |   |
|   |                                     | $\sigma_D^2 = 0$ | 0.656 <sub>[4,2,15]</sub>               |   |
|   | Modelled values.                    | $\sigma_S^2 = 0$ | 2.817 <sup>*</sup> <sub>[3,34]</sub>    |   |
|   |                                     |                  |   |   |

<sup>12</sup>Significance levels as in Table 4.

## Chapter 7

### Evidence for heterosis in resistance to *Ichthyophthirius multifiliis*.

#### 7.1. Introduction and aims.

When little or no additive genetic variance ( $V_A$ ) is present low or zero heritability values are obtained. In this situation selection is very likely to yield no gains. However, one component of the total phenotypic variance still remains which may allow genetical improvements to be made, that of genotypic variance due to dominance deviations ( $V_D$ ). This can be exploited via hybridization, which may produce heterosis in offspring, *i.e.* offspring which exceed either parent in the quality of a specific trait.

Hybridization can produce both useful and unusable offspring from any population. The reason why hybridization is so hit-or-miss lies in the fact that it exploits  $V_D$ , which is produced by the interaction of alleles at each locus. Dominance variance will, therefore, vary with genotype and is disrupted during meiosis. Consequently, dominance variance is not carried across generations, as with additive variance. The aim in any hybridization study, then, is to determine which two parental stocks, when hybridized, produce the desired offspring. The cross between parental stocks can then be repeated whenever such offspring are required (assuming inbred parental lines are used).

In the past there have been numerous reports of successful, and unsuccessful, hybridization programmes in the literature. The vast majority of these concentrate upon directly related economic traits, such as food conversion ratios, dressing percentage and size. Some have dealt with disease though. For example, Plumb *et al* (1975) found heterosis in resistance to channel catfish virus disease. However, many of these 'disease' studies deal

more with percentage survival rather than with a controlled specific disease outbreak.

In this study it was intended to look specifically for any evidence of heterosis in resistance to *I. multifiliis* under controlled infection procedures.

## 7.2. Hybrid production.

From the work of chapter 4 it was decided to perform crosses between the red platy fish (*X. maculatus*) and the sunset platy fish (*X. variatus*). These two stocks were the two which were separated the most, in terms of resistance to *I. multifiliis*, and which were also known to easily hybridize, which were breeding consistently. Reciprocal crosses between the two stocks were made and pure lines also maintained. Each breeding group involved four males and six virgin females. These ten fish were placed in a well planted 455mm x 255mm x 255mm aquarium, which was checked periodically for fry. Fry were removed and placed in 2.4 l tanks, where they were fed newly hatched *Artemia* nauplii. Progressively this diet was also supplemented with crushed Tetramin flakes, Promin pellets and Tubifex worms. As the fry grew they were moved onto a 455mm x 255mm x 255mm aquarium and then a 495mm x 670mm x 235mm aquarium.

A few problems were encountered in breeding the fish. The  $F_1$  hybrid fry were produced in abundance, with space being the limiting factor in rearing the offspring. The pure parental stocks bred less prolifically. However, sufficient numbers were obtained to enable infections to be carried out. The differences in fecundity became more pronounced in the next generation. For the second generation each  $F_1$  population was allowed to breed within its own group. Therefore, pure stocks and  $F_1 \times F_1$  fish, for each reciprocal hybrid, were produced. Very few pure stock fry were obtained in the second generation and differences also started to appear between the reciprocal hybrid stocks. Large numbers of *X. variatus* (sunset platy) male x *X. maculatus* (red platy) female fry were produced. However, the reverse cross  $F_2$  fry, whilst obtained in some number, were not as numerous.  $F_2$  fry were not exposed to *I. multifiliis* since there was insufficient time to raise them to maturity and to also infect them.

### 7.3. Infection protocol.

Infections were performed at three points in time (5/12/87, 15/12/87 and 30/12/87). On the first two occasions the two pure strains, the two reciprocal hybrids and *Ameca splendens* were infected. On the third occasion the pure *X. maculatus* (red platy) stock, the *X. maculatus* (red platy) male x *X. variatus* (sunset platy) female stock and *A. splendens* were infected. The reciprocal crosses act effectively as replicates, but could also give information on hybrid differences. Fish were allowed at least 24h to acclimatise to the infection tank. Ten fish were infected per tank, with the exception of *A. splendens* on the first occasion when only eight fish were infected. Ages at infection were : *X. maculatus* (red platy) 49.9 - 52.4 weeks, *X. variatus* (sunset platy) 46.7 - 47.1 weeks, *X. maculatus* male x *X. variatus* female 50.1 - 56.1 weeks, and *X. maculatus* female x *X. variatus* male, 51.7 - 52.6 weeks. The *A. splendens* fish were taken from a large population which were allowed to continually randomly reproduce. Ages at infection were, therefore, not quantified, but were estimated as approximately 52 weeks.

Infections were carried out as in chapter 3 at a dosage of 2000 theronts per fish (40 theronts per ml). *A. splendens* were also infected simultaneously to enable further comparisons to be made. Water quality was monitored prior to and upon termination of the experiment. All fish had never been exposed to *I. multifiliis* before and, in this investigation, only one isolate of parasite (isolate J) was used.

### 7.4. Results.

A total of 78 fish were successfully infected : 20 *X. maculatus* (red platy), 10 *X. variatus* (sunset platy), 20 *X. maculatus* male x *X. variatus* female hybrids, 10 *X. maculatus* female x *X. variatus* male hybrids and 18 *A. splendens*. Infections were unsuccessful on one occasion (15/12/87), with very few (<5) trophozoites per fish. Such fish were not used in any further experiments. The use of fish in this manner prevented further replicates of every genotype. Water quality was constant throughout the infections (temperature  $23.7 \pm 1.5^\circ\text{C}$ ; pH  $7.5 \pm 0.1$ ; nitrite  $0.01 \pm 0.005$  mg/l; nitrate  $4.2 \pm 0.7$  mg/l, ammonia  $0.3 \pm 0.1$  mg/l).

Surface area values varied from 2.58 to 7.49 cm<sup>2</sup> (mean = 4.58±1.02 cm<sup>2</sup>). *A. splendens* was noticeably much smaller in surface area (3.34±0.47 cm<sup>2</sup>) than any of the other species / hybrids used here. *X. variatus* (sunset platy) was the larger of the two parental species : *X. maculatus* (red platy) 4.68±0.78 cm<sup>2</sup>, *X. variatus* (sunset platy) 5.10±0.40 cm<sup>2</sup>. The two hybrids were of a similar size to *X. variatus* (sunset platy) : *X. maculatus* male x *X. variatus* female, 5.03±0.88 cm<sup>2</sup>, *X. maculatus* female x *X. variatus* male, 5.18±1.04 cm<sup>2</sup>. Female fish were significantly larger in surface area (4.81±1.01 cm<sup>2</sup>) than male fish (3.94±0.72 cm<sup>2</sup>,  $P < 0.001$ ). Hence, determination of surface area is important since this sexual difference in surface area may have otherwise have suggested a sexual difference in infection levels.

Results were analysed, as in chapter 3, using the Generalised Linear Interactive Modelling Package (GLIM) on a Prime computer. A model was fitted which took into account the following factors : time of the experiment, water temperature, sex of the fish, surface area of the fish and species of fish upon which the infection was recorded. When untransformed or square root transformed data were analysed plots of residual values against fitted values produced divergent distributions (Figure 34a and 34b). Analysis of variance requires such a distribution, which is an indication of the independence of the variance and the mean, to be random. A logarithmic transformation of total counts of parasites upon each fish produced such a random distribution (Figure 34c). The series of anova tables from this analysis are presented in Table 25.

The temperatures encountered here were 22.1 - 23.2°C for the first set of infections and 25.2 - 25.8°C for the second set of successful infections. The total temperature range of 3.7°C had no significant effect upon infection level ( $P > 0.10$ ).

Infection levels for the two sexes (all species and hybrids combined) were : males 117.8±118.2 trophozoites per fish, females 88.1±69.6 trophozoites per fish. These values were not significantly different ( $P > 0.10$ ) given variation due to all other factors. The effect of surface area was significant however



( $P < 0.05$ ). The GLIM package estimated that the relationship between surface area and infection level, given all other factors, was a factor of  $0.18 \pm 0.09$ . Hence, as suggested earlier, the extent of the surface area over which the infection is recorded is vitally important.

Significant differences occurred between the two times at which successful infections were performed ( $P < 0.01$ ). Mean parasite burdens for the two times were  $57.0 \pm 36.5$  and  $158.6 \pm 103.6$  trophozoites per fish. The first set of infections occurred in early December, whilst the second infections occurred in late December to early January. In addition to time effects, the difference in the two quoted mean infection levels is due to the fact that all species and hybrids were infected on the first occasion, but on the second occasion only *A. splendens*, *X. maculatus* (red platy) and *X. maculatus* male x *X. variatus* female fish were infected. *A. splendens* and *X. maculatus* (red platy) were the two most susceptible species and, hence, the infection levels on the second occasion will be higher, since these two species formed two-thirds of fish infected, as opposed to two-fifths of the fish infected on the first occasion.

Taking all the above factors into consideration, variation between species was significant ( $P < 0.001$ ). Ordering of the species from least to most resistant was as follows; *A. splendens*, *X. maculatus* (red platy), *X. variatus* (sunset platy), *X. xiphophorus variatus* hybrids (Figure 35). The two reciprocal hybrids did not differ significantly (*X. maculatus* male x *X. variatus* female  $46.0 \pm 24.5$  trophozoites per fish, *X. maculatus* female x *X. variatus* male  $27.2 \pm 6.9$  trophozoites per fish,  $P > 0.10$ ), whilst the *X. maculatus* (red platy) and *X. variatus* (sunset platy) were significantly different at the 5% level ( $125.8 \pm 76.7$  trophozoites per fish and  $49.0 \pm 25.9$  trophozoites per fish respectively). The two hybrid stocks differed from *X. variatus* (sunset platy) at the 1% level and all other pairwise comparisons were significant at the 0.1% level. Infection levels of *I. multifiliis* on the hybrids were only 42% of the mean parental level, demonstrating distinct heterosis. It is possible to calculate H, the degree of heterosis (Tave, 1986). This is simply the difference between the reciprocal  $F_1$  and parental populations, expressed as a percentage of the parental level.

Using the actual parasite counts a value of 16.2% is obtained here. Species-temperature interactions were not evident ( $P > 0.10$ ), suggesting that all species reacted in a similar manner over the temperature ranges of 3.7°C present here.

## 7.5. Discussion.

The effects of five factors upon infection levels were evaluated. The male : female ratio was 1 : 2.7, but variation due to sexual differences was not significant ( $P > 0.10$ ). The temperature range encountered was 3.7°C and this had no significant effect upon the infection level ( $P > 0.10$ ).

Variation due to surface area was significant ( $P < 0.05$ ). Female fish were significantly ( $P < 0.001$ ) larger ( $4.81 \pm 1.01 \text{ cm}^2$ ) than male fish ( $3.94 \pm 0.72 \text{ cm}^2$ ). Species differences in surface area were also seen. *A. splendens* was the smallest of the species used ( $3.34 \pm 0.47 \text{ cm}^2$ ), whilst *X. variatus* (sunset platy) and its hybrids were the largest fish ( $5.03 - 5.18 \pm 0.40 - 1.04 \text{ cm}^2$ ). The importance of evaluating the surface area over which the infection occurred is therefore stressed. The experimental protocol here permitted variation in these factors to be treated separately. Hence, species were compared, given variation due to differences in surface area, etc.

Time effects were also significant ( $P < 0.01$ ), despite the two infections only occurring twenty five days apart. The reason for this effect is unclear, since infection procedures were identical on each occasion. The same parasite isolate was used and parasite collection, incubation and exposure were identical. However, as mentioned previously, different strains / hybrids were infected at each time. Temporal variation will be discussed further in the final chapter.

Variation due to species was highly significant ( $P < 0.001$ ). *A. splendens* was the most susceptible species, followed by *X. maculatus* (red platy) and *X. variatus* (sunset platy). Pairwise comparisons showed that these species were all significantly different ( $P < 0.05$ ) from each other. Mean infection levels were 183.2 , 125.8 and 49.0 trophozoites per species respectively.<sup>5</sup> Heterosis was

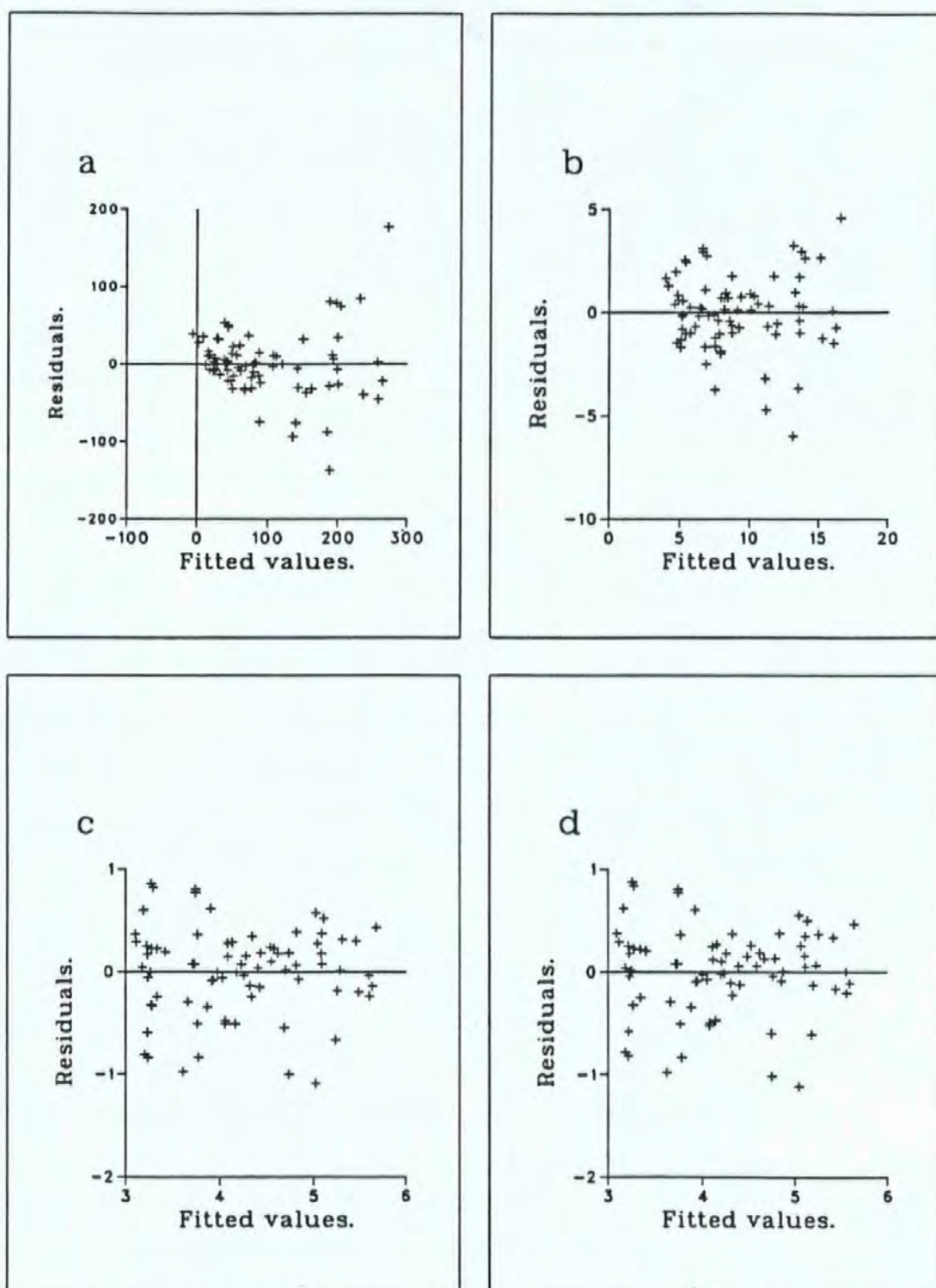
<sup>5</sup>Values are directly measured means, irrespective of surface area, etc.

distinctly observed in both of the reciprocal hybrids : *X. maculatus* male x *X. variatus* female, 46.0 trophozoites per fish, *X. maculatus* female x *X. variatus* male, 27.2 trophozoites per fish. Although these two hybrids differed significantly from both parental species and *A. splendens* ( $P < 0.01$ ) they were not significantly different from each other ( $P > 0.10$ ).

This heterosis to ichthyophthiriasis is significant in that it is the first case of such heterosis reported. It also confirms the genetic role of resistance to *I. multifiliis* infection. The usefulness of hybridization, however, is limited in that it can only be guaranteed for crossing the two parental stocks used. On the other hand, hybridization has the advantages of being much easier and less time consuming, than performing a within population selection program. This is especially important to the third world countries, where demands and limitations are much more acute.

The tropical species of fish were used here as model species, due to their short generation times and ease of handling in the laboratory. Since positive heterosis was obtained, the next stage would be to repeat this preliminary work on a commercial species, such as rainbow trout (*Salmo gairdneri*) or the common carp (*Cyprinus carpio*). Sövényi *et al* (1988) recently reported heterosis in resistance to *Aeromonas salmonicida* infection in carp hybrids, between an inbred Hungarian race of carp and a coloured race of Japanese carp. The results from this study and that of Sövényi *et al* (1988) suggest, therefore, that the scope for genetic gain in commercial species does exist.

Figure 34 Plots of residual values against fitted values after analysis for total *Ichthyophthirius multifiliis* burden on *Xiphophorus maculatus* (red platy), *Xiphophorus variatus* (sunset platy), the reciprocal hybrids between these two species, and *Ameoca splendens*. a. Untransformed data, b. Square root transformed data, c. Natural logarithmic transformed data, d. Natural logarithmic transformed data, species-temperature interaction residuals.

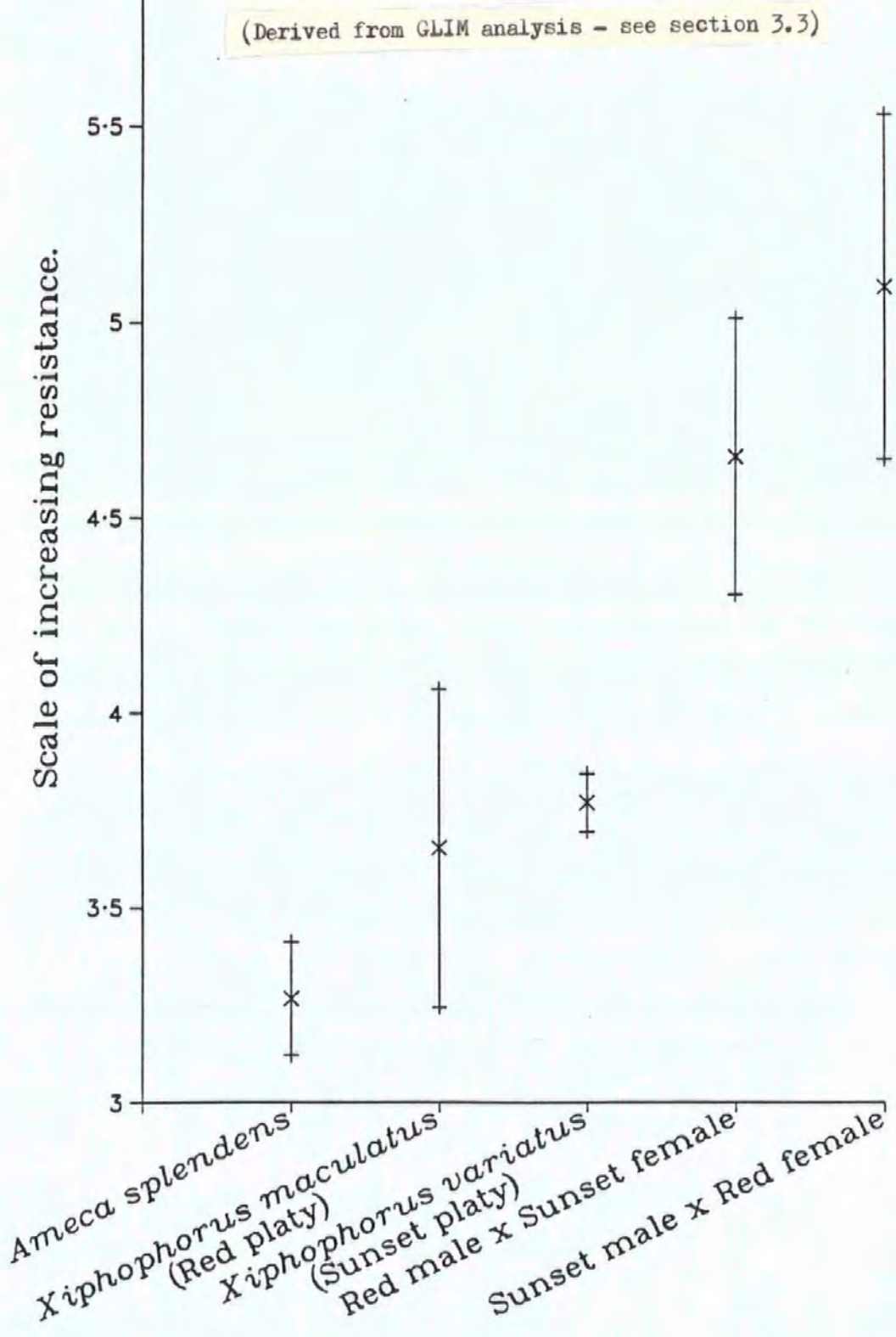


**Table 25** Analysis of variance tables for each of the variables in *Ichthyophthirius multifiliis* infections of pure *Xiphophorus maculatus* (red platy), pure *Xiphophorus variatus* (sunset platy), the two reciprocal hybrids of these species, and *Ameioba splendens*.<sup>13</sup>

| Source of variation.                                | d.f. | Sum of Squares. | Mean Squares. | F    | P    |
|---|------|-----------------|---------------|------|------|
| Effects due to time, species, temperature and sex.  | 7    | 43.1            | 6.2           | 30.6 | **** |
| Effects due to area.                                | 1    | 0.9             | 0.9           | 4.6  | **   |
| Residual.   | 69   | 13.9            | 0.2           |      |      |
| Total.  | 77   | 57.9            |               |      |      |
| Effects due to area, time, species and temperature. | 7    | 43.6            | 6.2           | 30.9 | **** |
| Effects due to sex.                                 | 1    | 0.4             | 0.4           | 2.1  | NS   |
| Residual.   | 69   | 13.9            | 0.2           |      |      |
| Total.  | 77   | 57.9            |               |      |      |
| Effects due to area, time, temperature and sex.     | 4    | 25.1            | 6.3           | 31.2 | **** |
| Effects due to species.                             | 4    | 18.9            | 4.7           | 23.4 | **** |
| Species - temperature interactions.                 | 1    | 0.05            | 0.05          | 0.25 | NS   |
| Residual.   | 69   | 13.9            | 0.2           |      |      |
| Total.  | 77   | 57.9            |               |      |      |
| Effects due to area, time, species and sex.         | 7    | 43.6            | 6.2           | 30.9 | **** |
| Effects due to temperature.                         | 1    | 0.4             | 0.4           | 2.1  | NS   |
| Residual.   | 69   | 13.9            | 0.2           |      |      |
| Total.  | 77   | 57.9            |               |      |      |
| Effects due to area, species, temperature and sex.  | 7    | 42.5            | 6.1           | 30.2 | **** |
| Effects due to time.                                | 1    | 1.5             | 1.5           | 7.3  | ***  |
| Residual.   | 69   | 13.9            | 0.2           |      |      |
| Total.  | 77   | 57.9            |               |      |      |

<sup>13</sup>Significance levels as in Table 4.

Figure 35 Variation in resistance to ichthyophthiriasis between *Xiphophorus maculatus* (red platy), *Xiphophorus variatus* (sunset platy), the reciprocal hybrids of these two species, and *Ameca splendens*. The y-axis scale corresponds to the co-efficients of resistance for each population, derived after analysis of the data for area, sex, temperature and time.



## Chapter 8

### A discussion of the genetics of susceptibility to *Ichthyophthirius multifiliis* infection in fish.

#### 8.1. Introduction.

In the introductory chapter of this work the impact of disease upon aquaculture production was discussed, as were the available methods of prevention and cure of disease. It was stated that prevention, especially through genetical approaches, may be preferable to curing diseases as they occur. The following chapters then assessed various genetical aspects of susceptibility to ichthyophthiriasis.

In these studies several variables were incorporated into the analysis. Some variables were unique to each particular study, whilst some were common throughout. Table 26 shows a summary of the significance of each variable throughout the studies here.

#### 8.2. Variation with time of the experiment.

The level of infection was found to fluctuate, in all of the studies, with the time of year at which infection occurred. This was even evident in the *Xiphophorus maculatus* yellow comet tail heritability study, where infections were only separated by 9 days. Infections were performed at various points throughout the year, over a three year period (Figure 36). The infections of *Ameioba splendens*, with four parasite exposure doses, in chapter 3, occurred throughout May, June and early July. In the species / strains of fish comparisons infection levels rose from mid-February to a peak in late April. Much narrower peaks occurred in July, late September / early October and November. In the between genotype infections of carp (*Cyprinus carpio*) infections were unsuccessful

during April, but sizeable infection levels were obtained during May. Following this, infections were only successful in mid-June and early August. Therefore, there appears to be a conglomeration of successful infections in the spring period, with occasional isolated peaks occurring after this time (Figure 36).

The three heritability studies involved infections at isolated points throughout the year, rather than a series of infections covering several months. Out of the three species the highest infection levels, of 84 to 597 trophozoites on a single fish, were found on the *X. maculatus* yellow comet tail. These infections occurred in late September and mid-October. On *X. maculatus* Vera cruz infection levels of upto 246 trophozoites were obtained on fish infected in late May. The *A. splendens* infections, on the other hand, only realised infection levels of upto 30 trophozoites from infections which occurred in mid-January. The heterosis study involved infections in December, with higher infection levels at the end of the month (449 trophozoites) than at the start of the month (136 trophozoites).

The three years of data shown in Figure 36 have been pooled together and is shown in Figure 37. It should be noted that the two large peaks of September and October occur very close together, in late September and early October. Hence, these two bars of the histogram can, and should, be treated as one, since the first two-thirds of September and the second half of October provided no successful infections.

Meyer (1970) reported seasonal outbreaks in *Trichodina*, *I. multifiliis*, *Plistophora*, *Costia*, *Gyrodactylus*, *Dactylogyrus*, *Cleidodiscus* and *Lernaea* over the 1963 - 1968 period. Overall greatest incidence of disease occurred in April (19.4% of all reported disease outbreaks), with the period of March to July inclusive covering 72.1% of all disease outbreaks. More specifically, April was also the month with the greatest number of ichthyophthiriasis outbreaks (20.7%). January to April inclusive encompassed 72.4% of *I. multifiliis* outbreaks. The remaining 27.6% of *I. multifiliis* outbreaks were spread over the 8 months of May to December, with two minor peaks of 8.6% each in May and



November accounting for most of these outbreaks. This annual cycle for *I. multifiliis* is constructed from the data presented by Meyer (1970) in Figure 38.

When interpreting Figures 37 and 38 it must be remembered that the former includes data over 3 years and the latter data from 5 years of records. The data are also presented in a slightly different format - maximum infection level vs. percentage of ichthyophthiriasis outbreaks. However, both studies give an indication of the fluctuations in the extent of ichthyophthiriasis over the year.

A very similar situation was, therefore, reported by Meyer (1970) to that found here. Major incidence and severity of the disease occurs in April. High incidence of the disease in the early part of the year is coupled to a low level of severity in September-October and December is coupled to a low level of incidence. However, the discussion here of the reasons for such an annual distribution cannot be similar to Meyer (1970) who argued that climbing water temperatures were responsible for the high incidence of *I. multifiliis* in the early part of the year. This was also coupled to increased handling stress, particularly in April. The much lower incidence levels later in the year were explained by low water temperatures retarding the parasite growth. Another possible cause is that the majority of fish had received an infection of *I. multifiliis* and their immunity was now building up, thus reducing the incidence of ichthyophthiriasis. In this study, though, temperatures were relatively constant, with a typical standard deviation of 1.0°C. In addition, there was no particular point in the year when handling stresses will have been greater and all infections occurred upon fish which had never been infected with *I. multifiliis* before. A similar temporal variation to the one found here was reported by Yamaguchi *et al* (1980), where the humoral response of trout (*Salmo gairdneri*) was found to depend upon season, even when temperature was kept constant. MacLennan (1935b) also recorded seasonal variations in the incidence of ichthyophthiriasis, as did Parker (1965), Migala (1971) and Lahav & Sarig (1973). An explanation of this effect was not provided, nor is one apparent in this study.

It is unclear whether the annual variation here is due to fluctuations in the host or the parasite. There is some subjective evidence that fluctuations within the parasite are involved, since periods of low or no infection can be linked to periods of difficulty or failure in maintenance of the parasite stocks. However, this does not rule out the possibility of annual fluctuations within the host also. Further investigation of this relationship is required to permit a greater discussion of a most interesting phenomenon. For the purposes of further discussion in this chapter it is important to remember that this temporal effect was incorporated into the analysis model, and is, therefore, not a confounding issue.

### 8.3. Temperature and tank effects.

In five of the seven analyses where temperature was included, the effect was not significant ( $P > 0.10$ ). The two studies where temperature did form a significant amount of the variation are, for the between genotype investigation, using carp (*C. carpio*), and the strain / species of fish comparisons. In the carp investigation three temperature regimes were used (16, 19 and 24°C), resulting in a large range of temperatures being covered and the significant differences observed are therefore not surprising. The variation in temperature in the species / strains of fish experiments was much less ( $24.1 \pm 1.0^\circ\text{C}$ ) however.

In the tank effect experiment four identical infections were performed using *A. splendens*. Significant ( $P < 0.001$ ) variation was observed due to tank effects - the uniqueness of each aquarium. Closer examination, however, using pairwise comparisons, demonstrated that only one tank differed from the other three tanks, which formed an homologous group.

The reason for discussing temperature and tank effects together is that the two are inseparably linked. In the tank effect experiment each tank was assigned an identification number. Temperatures were recorded for each tank in the other studies. Hence, a tank could be identified by either an assigned number, or its' temperature. The two values do not distinguish any other difference than between aquaria. It therefore follows, that the two effects are

synonymous. A temperature effect which is not significant also implies the absence of any tank effects. A significant temperature effect, on the other hand, suggests the presence of a tank effect, of which temperature may, or may not, be implicated in the cause. The important point, though, is that the tank effect was taken into account in the analysis model. The variance due to tank effect was, therefore, partitioned off from the variance of interest.

#### 8.4. Sex and surface area effects.

These two sources of variation are discussed together, not because they are synonymous, but because there is a suggestion of some connection between the two effects. Variation due to surface area was significant ( $P < 0.10$ ) in every study except the heritability determinations of *X. maculatus* (Vera Cruz) and *A. splendens*. On the other hand, variation due to sex was not significant ( $P > 0.10$ ) in every study except the same two heritability investigations. Indeed, a further point of interest is that variation due to sex was not significant in the *A. splendens* tank effect investigation, yet it was significant in the *A. splendens* heritability determination study.

It has been suggested that stress can affect a fish's resistance to infection (Snieszko, 1974; Wedemeyer & Wood, 1974; Wedemeyer *et al*, 1976). It is also known that population density influences the age and weight at sexual maturity (Kallman & Borkoski, 1978). The *A. splendens* for the tank effect experiment came from a large, random breeding population kept in the Polytechnic aquarium. Here the density of fish is higher than in the heritability study, where individual broods were reared separately. It is not unreasonable then to propose that stress levels were higher in the Polytechnic aquarium derived fish, than in the heritability determination fish and also that a similar relationship may have occurred in the other stocks of fish. Since larger fish are, generally, able to adapt to stress better, in the presence of stress, surface area (or size) is a significant factor in the susceptibility to *I. multifiliis* infection. When stress due to high population densities is removed, the role of sexual differences then appears to come into play.

Houghton & Matthews (1986) found that elevated levels of adrenocorticotrophic hormones in fish populations, such as those which occur during stress, could lower resistance to disease, and in particular ichthyophthiriasis. Hence, there appears to be a correlation between stress, such as that brought about through stocking levels, and disease resistance.

### **8.5. Parasite strain, parasite isolate and parasite exposure levels.**

In the between genotype study on carp two different strains of *I. multifiliis* were used. One of these strains arose from fish imported from Singapore, whilst the other was obtained from a Devonshire fish farm. The analysis did not find any significant difference in the relative infection levels of these two parasite strains. However, with respect to changing temperatures the two strains did differ. The level of infection rose with increasing temperature for the tropical parasite strain, whilst it *decreased* with increasing temperature for the temperate parasite strain. The point was made that this effect illustrates that higher infection levels occurred at temperatures which were closer to the parasite strains' acclimation temperature. The lack of success in attempting to reverse the passage temperature for each strain was taken as evidence for differences between the adaptation of the two strains. Nigrelli *et al* (1976) report similar results when attempting to culture mature tropical *I. multifiliis* at 4°C. Parasites did not divide beyond the eight cell stage and all parasites died within 9 days. Subasinghe & Sommerville (1985) found no difference in the reproductive capabilities of two strains of *I. multifiliis* from "two distinctly different geographic localities". However, no mention is made of the infective capabilities of the two parasite strains.

Such a strain difference would explain other observed phenomena. In particular, there is some debate about the effectiveness of increased temperature in controlling ichthyophthiriasis (Ghadially, 1964b; Cross, 1972; Richards, 1977; Leibovitz, 1980). Sometimes such an action cures the disease, yet sometimes it only serves to enhance the situation. Conclusive evidence of strain differences is still required therefore.

One approach which would yield much useful information would be to transfer fish to different temperatures at several intervals after initial infection. Observations upon the infection levels would determine the extent and significance of temperature changes upon infection. Performed with both parasite strains this would present more information concerning the different parasite-strain temperature responses.

The extent of differences in parasite isolate were even more difficult to determine conclusively, due to extrinsic aliasing within the data set. However, species-parasite isolate interaction terms did provide some information. *A. splendens* and *X. maculatus* (red platy) were infected on two occasions, but with different parasite isolates. On one occasion *A. splendens* was the most susceptible fish, whilst on the second occasion the reverse was true. Hence, the relative infection levels on the two species appeared to depend upon the parasite isolate used.

The effect of increasing the exposure level to the parasite upon infection level was also significant. Exposure levels of 2000 3000, 6,000 and 12000 theronts per fish were used. These are relatively low levels since an individual cyst may produce upto 2000 theronts (Prytherch, 1924 ; Butcher, 1941 ; Meyer, 1974). The number of theronts produced per cyst does appear to vary considerably though. Hence, the use of theronts to standardise the exposure levels is vitally important since 6 cysts per fish could well yield 2000 or 12000 theronts.

The relevance of parasite strain and parasite isolate differences to aquaculture is clear. It has already been mentioned that relative susceptibilities of fish species can reverse with parasite isolate changes. Therefore, if resistant strains of fish were established there is a possibility that such fish would be very susceptible to infection in a different environment. This effect is reminiscent of resistance to bacterial strains, which is the result of different antigen sites or protein structures. Thus resistance to one bacterial strain does not necessarily confer resistance to all bacterial strains of the same species. It would be interesting to see what an extensive immunogenetic study on susceptibility to *I. multifiliis* would produce.

Perhaps, above all, the results discussed in this section stress the need to establish recognised strains of *I. multifiliis* for research purposes. At present this is precluded by the problems of freeze storage of ciliates. Further work in this area would be a milestone in unravelling the complexities of *I. multifiliis* infection dynamics.

### **8.6. Fish strain / species / genotype differences.**

Initially approaches were made to evaluate the presence of any within and between species differences in susceptibility to *I. multifiliis* infection. Such differences were found within *X. maculatus* and between this species and *X. variatus*, *A. splendens* and *Ilyodon xanthusi*. Between genotype differences in the carp, between four recognised scale pattern types, provided clear evidence of within species between genotype variation in susceptibility. Fully scaled carp were found to be more resistant than any of the three mirror scale types of carp. Further to this, within population studies attempted to quantify the extent of additive variance, which could be exploited via selection. These studies adequately demonstrated that there is considerable variation in heritability values, but with some promisingly high values being recorded (0.75 for *X. maculatus*, Vera Cruz). Between population studies also demonstrated distinct heterosis in susceptibility for *X. maculatus* (red platy) x *X. variatus* (sunset platy) hybrids. Infection levels of *I. multifiliis* in hybrids were only 42% of the mean parental value.

The aim of this study was to assess the extent of genetic variation in susceptibility to ichthyophthiriasis. This has been suggested in the past (*e.g.* Parker, 1965; McCallum, 1982, 1986; McLay, 1985), but never examined in detail. Recently Ilyassov (1987) presented a review of the genetic principles of disease resistance in fish. Clearly genetic variation in susceptibility exists and the way is now open to pursue such work further. Although differences in susceptibility were established here the molecular nature of these differences was not investigated. Madhavi & Anderson (1985) identified resistant and susceptible guppy fish (*Poecilia reticulata*) to infection with *Gyrodactylus*

*bullatarudis*, but were also unable to explain the mechanism of resistance. However, cellular responses were suggested. Wahli *et al* (1985, 1986) found evidence that increased ascorbic acid levels promoted protection against *I. multifiliis*, but were again unable to explain the mechanism for protection. In the between genotype study of carp here mention was made that the resistance of fully scaled carp may be due to the physical integrity of the scale covering or to some other fundamental molecular difference. Further comparisons were suggested there, to yield more information on this matter.

Since the presence of genetic variation has been demonstrated and a suitable method of analysing the data determined, another approach would be to evaluate the number of genes involved. This can be obtained by performing a diallele cross analysis. Such an analysis provides a large amount of information, including estimates of maternal, additive and dominance variance. To obtain so much detail, however, requires many crosses to be performed, with resulting practical problems, particularly of space. A full diallele cross involves replicated, reciprocal crosses between several populations. Hence, a diallele study using 6 populations of fish would produce 36 broods for each replicate. Due to practical problems several modifications of the diallele study have been derived, *e.g.* the replicated non-reciprocal study.

The carp study here also highlighted the importance of gene-environment interactions. Indeed, the point was made that if carp were not reared under ideal conditions the differences between the four scale types may have been greater. The possible extent of gene-environment interactions are endless, yet it is through studies such as this that an awareness of particular interactions can be developed. Such an awareness is essential if the aquaculture industry is to realise the extent and limitations of genetically selected disease resistant stocks. A clear understanding of these boundaries by all concerned (*i.e.* geneticists and fish farmers) will provide firm ground upon which genetic potential for increased disease resistance can be realised.

In summary, then, there is good potential, for increased resistance to

ichthyophthiriasis in fish stocks, through either selection or hybridization. There is, perhaps understandably, some reticence to support such approaches on a commercial scale. In general, benefits are not normally immediate, nor are they guaranteed. This initial study has proven that further work *is* likely to be beneficial. In the short-term benefits could be reaped from hybridization programs, whilst, on a long-term basis, selection would provide more secure benefits. It is strongly recommended, therefore, that a pilot program be commenced using commercially important species, such as trout and salmon. Such an investment would promote a more competitive industry, from reduced losses and treatment costs, and also a healthier, and therefore better, product.



Table 26 Summary table of each of the variables in the analyses of this study.<sup>14</sup>

|  | F <sub>[d.f.]</sub> <sup>P</sup> | Comments                    |
|--|----------------------------------|-----------------------------|
| Time of experiment.                                  |                                  |                             |
| <i>Ameca splendens</i> dose experiment.              | 13.24 <sup>****</sup><br>[2,77]  | 63 days.                    |
| Species/strains differences.                         | 93.63 <sup>****</sup><br>[8,255] | 294 days.                   |
| Carp experiment.                                     | 17.38 <sup>****</sup><br>[5,205] | 122 days.                   |
| <i>X. maculatus</i> yellow comet tail h <sup>2</sup> | 26.66 <sup>****</sup><br>[1,55]  | 18 days.                    |
| <i>X. maculatus</i> Vera Cruz h <sup>2</sup>         | 83.48 <sup>****</sup><br>[1,84]  | 9 days.                     |
| Hybrid experiment.                                   | 7.30 <sup>**</sup><br>[1,69]     | 25 days.                    |
| Temperature.   |                                  |                             |
| <i>Ameca splendens</i> dose experiment.              | 0.27 <sup>NS</sup><br>[1,77]     | 24.0 ± 1.0°C                |
| Species/strain differences.                          | 5.30 <sup>**</sup><br>[1,255]    | 24.1 ± 1.0°C                |
| Carp experiment.                                     | 6.06 <sup>**</sup><br>[1,205]    | 20.5 ± 3.2°C                |
| <i>X. maculatus</i> yellow comet tail h <sup>2</sup> | 1.71 <sup>NS</sup><br>[1,55]     | 21.8 ± 0.2°C                |
| <i>X. maculatus</i> Vera Cruz h <sup>2</sup>         | 1.42 <sup>NS</sup><br>[1,84]     | 25.2 ± 0.3°C                |
| <i>A. splendens</i> h <sup>2</sup>                   | 0.84 <sup>NS</sup><br>[1,38]     | 24.8 ± 0.4°C                |
| Hybrid experiment.                                   | 2.10 <sup>NS</sup><br>[1,69]     | 23.7 ± 1.5°C                |
| Tank effect.   |                                  |                             |
| <i>Ameca splendens</i> dose experiment.              | 13.40 <sup>****</sup><br>[3,34]  | 4 identical tanks infected. |
| Sex (Male : Female numbers)                          |                                  |                             |
| <i>Ameca splendens</i> tank effect experiment.       | 1.31 <sup>NS</sup><br>[1,34]     | 1 : 5                       |
| Species/strains differences.                         | 0.18 <sup>NS</sup><br>[1,255]    | 1 : 1.8                     |
| <i>X. maculatus</i> yellow comet tail h <sup>2</sup> | 0.05 <sup>NS</sup><br>[1,55]     | 1 : 1.1                     |
| <i>X. maculatus</i> Vera Cruz h <sup>2</sup>         | 2.83 <sup>*</sup><br>[1,84]      | 1 : 1.3                     |
| <i>A. splendens</i> h <sup>2</sup>                   | 6.13 <sup>**</sup><br>[1,38]     | 1 : 1.8                     |
| Hybrid experiment.                                   | 2.10 <sup>NS</sup><br>[1,69]     | 1 : 2.7                     |

<sup>14</sup>P = probability value [see Table 4] and d.f. = degrees of freedom.

Table 26 continued ...

|  | F <sub>[d.f.]</sub> <sup>p</sup> | Comments   |
|--|----------------------------------|--|
| <b>Surface area.</b>                                 |                                  |  |
| <i>Ameca splendens</i>                               | 31.78 ****<br>[1,77]             | 3.43 ± 0.88 cm <sup>2</sup>                        |
| <i>Ameca splendens</i> tank effect experiment.       | 43.76 ****<br>[1,34]             | 2.73 ± 0.69 cm <sup>2</sup>                        |
| Species/strains differences.                         | 38.10 ****<br>[1,255]            | 4.94 ± 2.05 cm <sup>2</sup>                        |
| Carp experiment.                                     | 38.14 ****<br>[1,205]            | 3.88 ± 1.51 cm <sup>2</sup>                        |
| <i>X. maculatus</i> yellow comet tail h <sup>2</sup> | 6.25 **<br>[1,55]                | 6.11 ± 1.40 cm <sup>2</sup>                        |
| <i>X. maculatus</i> Vera Cruz h <sup>2</sup>         | 2.41 **<br>[1,84]                | 2.29 ± 0.79 cm <sup>2</sup>                        |
| <i>A. splendens</i> h <sup>2</sup>                   | 0.04 **<br>[1,38]                | 5.68 ± 1.59 cm <sup>2</sup>                        |
| Hybrid experiment.                                   | 4.60 **<br>[1,69]                | 4.58 ± 1.02 cm <sup>2</sup>                        |
| <b>Parasite strain.</b>                              |                                  |  |
| Carp experiment.                                     | 2.55 **<br>[1,205]               | 2 strains used.                                    |
| <b>Parasite isolate.</b>                             |                                  |  |
| Species/strains differences.                         | Extrinsically aliased.           | 4 tropical parasite isolates used.                 |
| <b>Parasite exposure level.</b>                      |                                  |  |
| <i>Ameca splendens</i> dose experiment.              | 163.82 ****<br>[1,77]            | 2000, 3000, 6000 and 12000 theronts per fish used. |
| <b>Strain / species of fish.</b>                     |                                  |  |
| Species/strains differences.                         | 18.50 ****<br>[6,255]            | 8 strains / species used.                          |
| Hybrid experiment.                                   | 23.45 ****<br>[4,69]             | 3 species & 2 hybrids used.                        |
| <b>Genotype (carp scale patterns)</b>                |                                  |  |
| Carp experiment.                                     | 10.95 ****<br>[3,205]            | 4 scale types of carp used.                        |

**Figure 36** Summary histograms of monthly maximum infection levels from this study (1985-88) where an exposure dose of 2,000 theronts per fish was used.

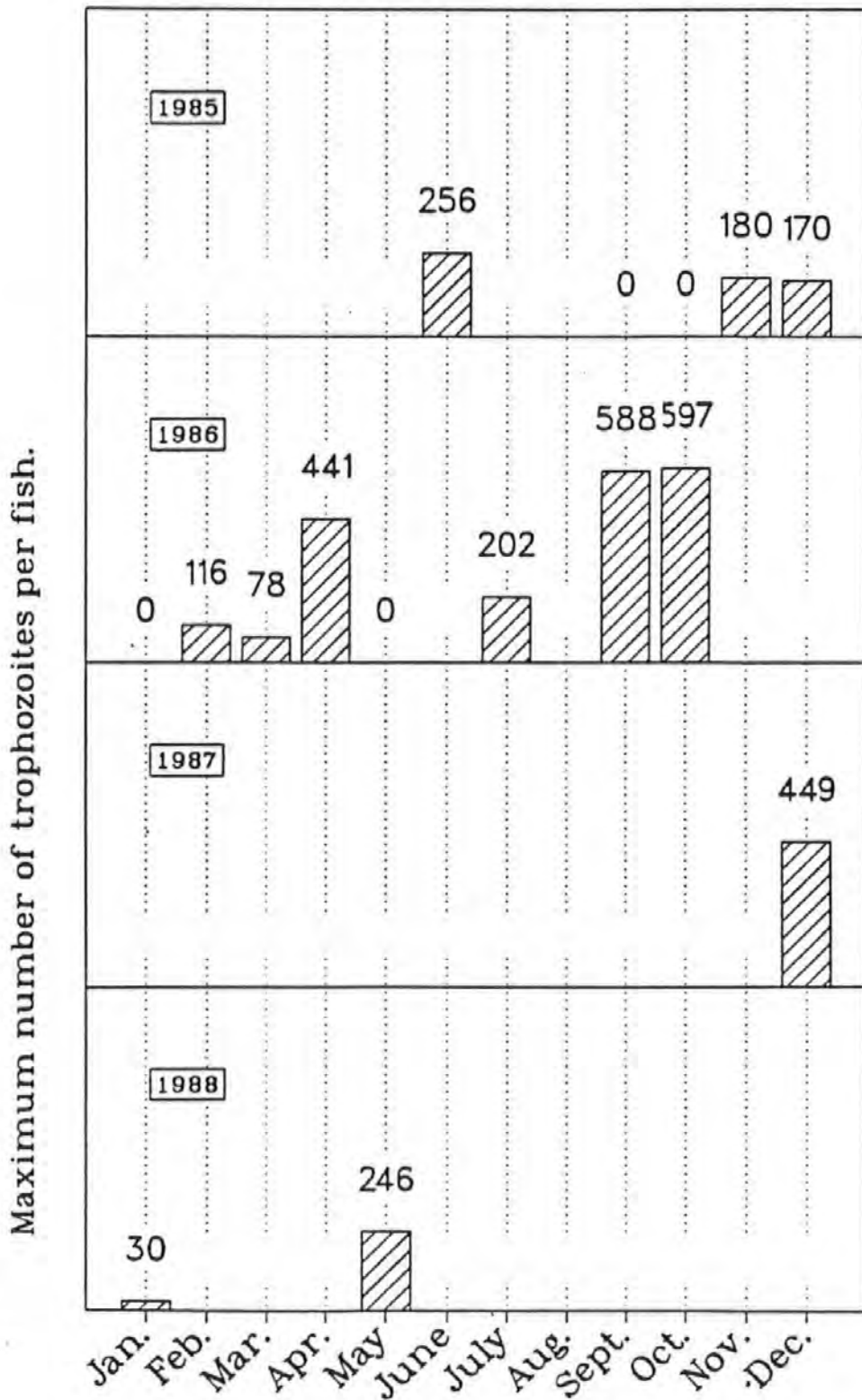


Figure 37 Overall summary histogram for 1985-88.

for Ichthyophthirius multifiliis infections  
(dose level of 2000 theronts per fish used  
throughout).

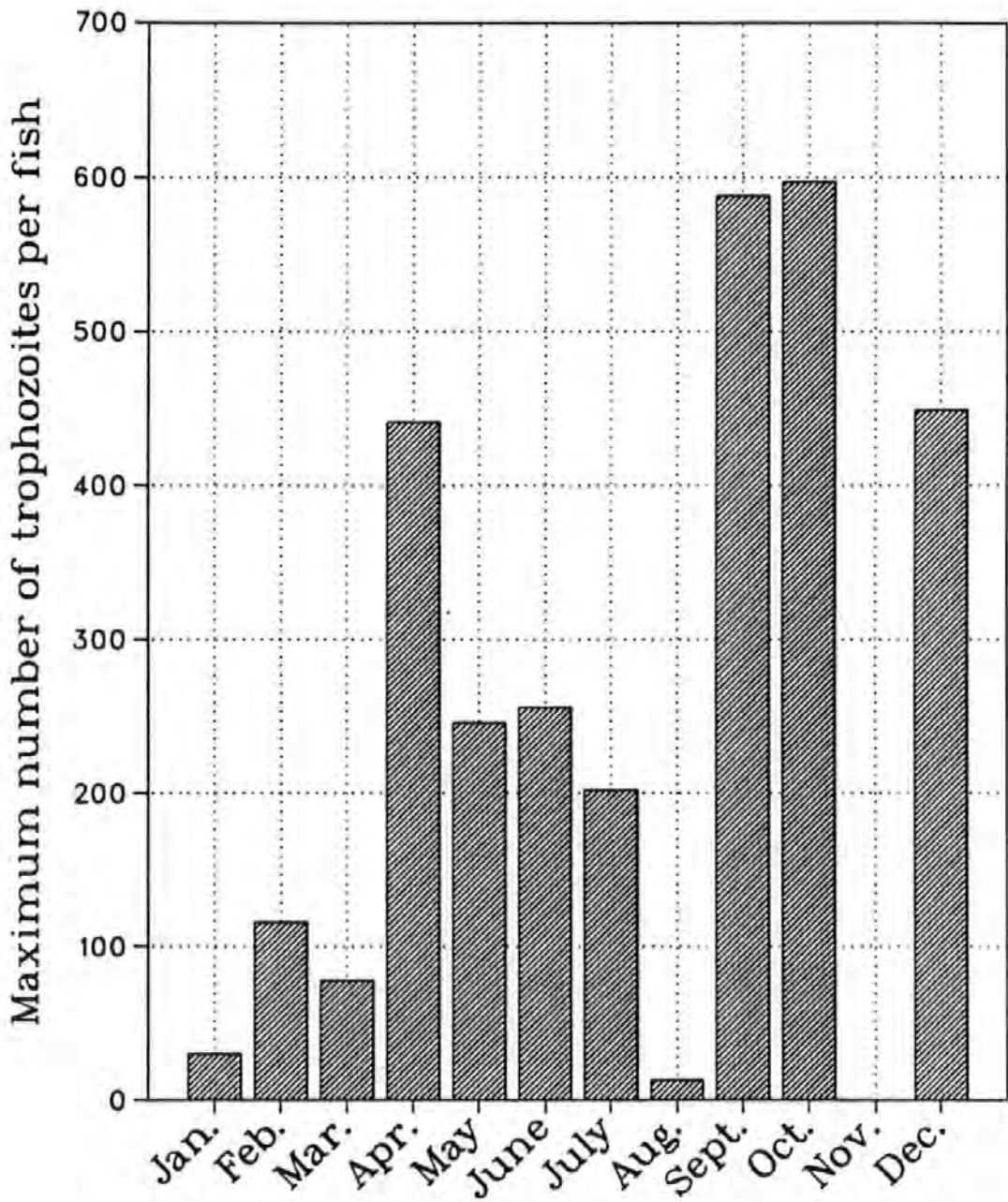
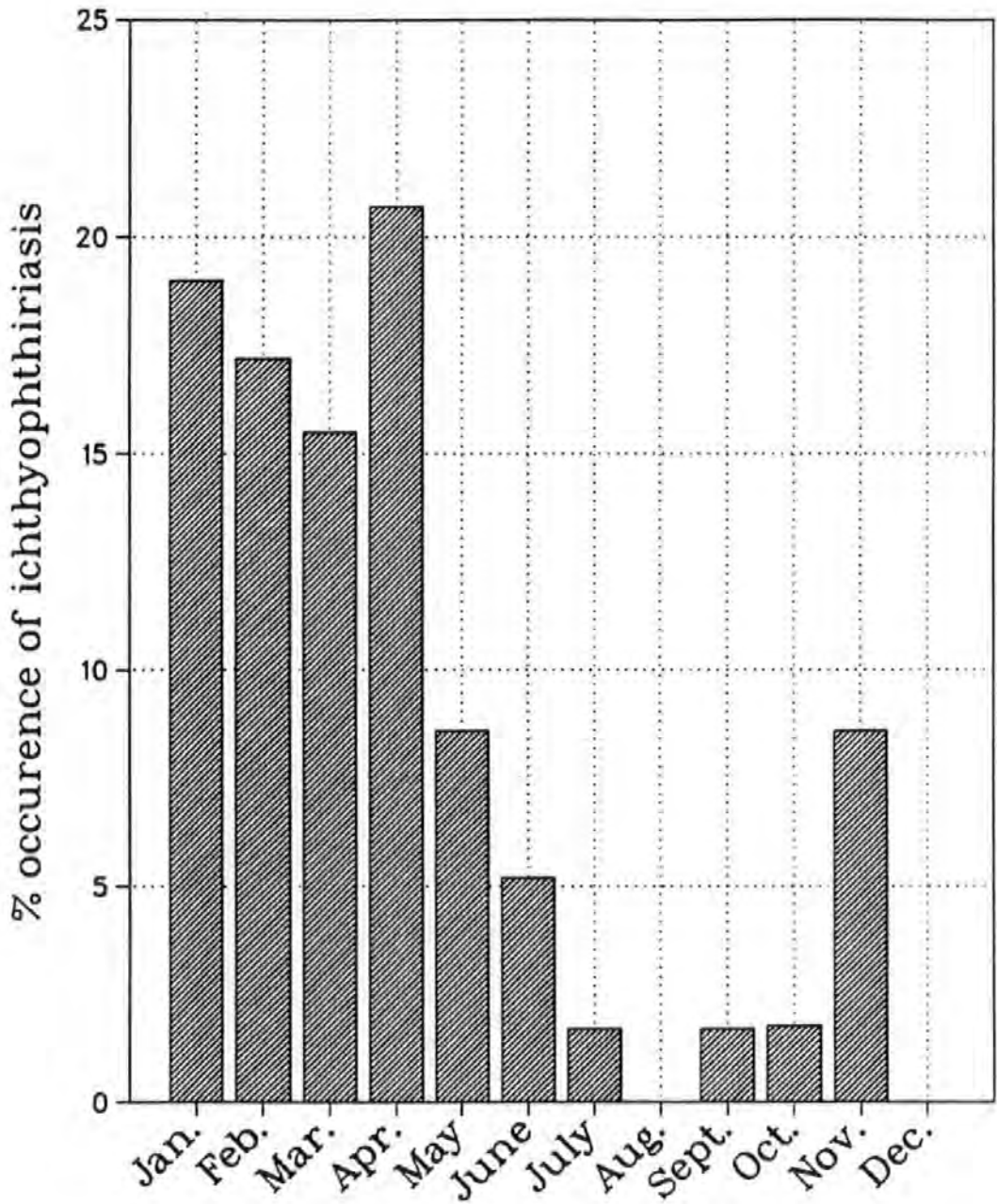


Figure 38 Annual variance in occurrence of ichthyophthiriasis, (redrawn from data of Meyer, 1970).



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## **Appendix A**

**Suite of five BASIC programmes for evaluation of fish  
body surface area measurements.**

**A.1. BBC BASIC programme for the entering of body  
circumference measurements.**



```

10REM PROGRAMME a - B. ENTER
20REM ENTERING OF NO. OF DATA VALUES, SL, TL &
  ACTUAL DATA VALUES FOR BODY CIRCUMFERANCE
  AREAS
30ONERROR PRINTERL:PRINTERR:END
40MODE7
50CLOSE£0
60DIM DV(200)
70*.
80INPUT"ENTER FILENAME IN WHICH TO STORE DATA "
  FILE$
90C=OPENOUT(FILE$)
100PRINT''
110INPUT"Enter no. of data values "N
120INPUT"Enter SL "SL
130INPUT"Enter TL "TL
140FOR X%=1 TO N
150PRINT"Enter data value ";X%
160INPUTDV(X%)
170NEXT
180CLS
190PRINT"N = ";N
200PRINT"SL = ";SL
210PRINT"TL = ";TL
220FOR X%=1 TO N
230PRINTDV(X%)
240NEXT
250PRINT"Q FOR QUIT":PRINT"R FOR RE-ENTER DATA":
  PRINT"S FOR SAVE DATA AND CONTINUE"
260REPEAT
270A$=GET$
280UNTIL INSTR("QRSqrs",A$)
290IF A$="Q" OR A$="q" CLOSE£0:PROCEND
300IF A$="R" OR A$="r" GOTO 100
310IF A$="S" OR A$="s" PROCWRITE:CLS:GOTO 100
320END
330
340DEFPROCWRITE
350PRINT£C,N,SL,TL
360FOR X%=1 TO N
370PRINT£C,DV(X%)
380NEXT
390ENDPROC
400
410DEFPROCEND
420*.2
430REPEAT:PRINT"CONTINUE OR DELETE FILES C/D ?":
  A$=GET$:UNTIL A$="C" OR A$="c" OR A$="D"
  OR A$="d"
440IF A$="D" OR A$="d" THEN CLS:*DR.2:*.:END
450CHAIN"FFAREAD"

```

460ENDPROC

**A.2. BBC BASIC programme to read data for fin areas transferred from Prime mainframe computer via the file transfer protocol, Kermit.**

```

10REM PROGRAMME b - NAME = 'FFAREAD'
20REM READS FIN DATA TRANSFERRED FROM
    PRIME MAINFRAME.
30CLOSE£0
40ONERROR CLOSE£0:PRINT"CHAINING FBAREAD":
    CHAIN"FBAREAD"
50MODE0
60*.
70INPUT"ENTER FILENAME FOR FIN DATA FILE
    TRANSFERRED FROM PRIME "FILE$
80D=OPENUP (FILE$)
90A=OPENOUT ("FA.OUT")
100DIM S$(10),A(15)
110N%=1:S%=1:F%=3:Z%=13
120FOR X%=1 TO F%
130PROCREAD
140PROCSAVES
150NEXT
160FOR X%=1 TO 3
170PRINT£A,S$(X%)
180PRINTS$(X%)
190NEXT
200FOR X%=1 TO Z%
210PROCREAD
220PROCSAVES
230NEXT
240PROCCALC
250PROCWRITE
260PROCREAD
270PROCSAVES
280IF LEFT$(S$(S%-1),4)="FISH" THEN N%=1:Z%=12:
    S%=5:PROCMOVE:GOTO 200
290IF LEFT$(S$(S%-1),4)<>"FISH" THEN
    S$(1)=S$(S%-1):PRINT£A,"*****":
    PRINT"*****":PROCCLR:PROCCLRS:
    F%=2:Z%=13:S%=2:N%=1:GOTO 120
300CLOSE£0
310END
320
330DEFPROCREAD
340REPEAT
350I=BGET£D
360IF I<>13 A$=A$+CHR$(I)
370UNTIL I=13
380ENDPROC
390
400DEFPROCSAVES
410IF LEFT$(A$,1)="+" THEN L=LEN(A$):
    A$=RIGHT$(A$,L-1):A(N%)=VAL(A$):
    N%=N%+1:GOTO430
420IF LEFT$(A$,1)<>"+" THEN S$(S%)=A$:S%=S%+1

```

```

430A$=""
440ENDPROC
450
460DEFPROCCLR
470A$=""
480FOR Q%=1 TO 15
490A(Q%)=0
500NEXT
510ENDPROC
520
530DEFPROCCALC
540TDF=0:TCF=0:TAF=0:MDF=0:MCF=0:MAF=0:SDD=0:
    SDC=0:SDA=0
550FOR X%=1 TO 3
560TDF=TDF+A(X%)
570TCF=TCF+A(X%+3)
580TAF=TAF+A(X%+6)
590NEXT
600MDF=TDF/3
610MCF=TCF/3
620MAF=TAF/3
630FOR X%=1 TO 3
640SDD=SQR((A(X%)-MDF)*(A(X%)-MDF)/2)+SDD
650SDC=SQR((A(X%+3)-MCF)*(A(X%+3)-MCF)/2)+SDC
660SDA=SQR((A(X%+6)-MAF)*(A(X%+6)-MAF)/2)+SDA
670NEXT
680ENDPROC
690
700DEFPROCWRITE
710PRINT#LA,S$(4),MDF,MCF,MAF,SDD,SDC,SDA
720PRINTS$(4):PRINTMDF:PRINTMCF:PRINTMAF:
    PRINTSDD:PRINTSDC:PRINTSDA
730ENDPROC
740
750DEFPROCCLRS
760FOR X%=2 TO 10
770S$(X%)=""
780NEXT
790ENDPROC
800
810DEFPROCMOVE
820IF LEFT$(S$(8),4)="FISH" THEN S$(4)=S$(8)
830PROCCLR
840ENDPROC

```

**A.3. Optional BBC BASIC programme to add extra data values to those already entered, if required.**

```

10REM OPTIONAL PROGRAMME - B.ADD
20REM USED TO ADD DATA TO A PREVIOUSLY CREATED
30REM FILE IF DATA ENTRY SESSION NOT COMPLETED.
40CLOSE#0
50DIMD(15)
60B=OPENUP("B.81287")
70REPEAT
80INPUT#B,N,SL,TL
90PRINT"N =";N;" SL = ";SL;" TL =";TL
100FOR I=1 TO N
110INPUT#B,A
120PRINTA
130NEXT I
140UNTIL EOF#B=-1
150PRINT"END OF FILE - ENTER MORE DATA Y/N?"
160REPEAT
170A$=GET$
180UNTIL A$="Y" OR A$="N"
190IF A$="N" END
200INPUT"ENTER NUMBER OF VALUES "N
210INPUT"ENTER SL "SL
220INPUT"ENTER TL "TL
230FOR L=1 TO N
240INPUT"ENTER DATE VALUE "D(L)
250NEXT
260PRINT"N = ";N:PRINT"SL = ";SL:PRINT"TL = ";TL
270FOR L=1 TO N:PRINTD(L):NEXT
280REPEAT
290PRINT"RETYPE - R"
300PRINT"SAVE --- S"
310A$=GET$
320UNTIL A$="S" OR A$="R"
330PRINT#B,N,SL,TL
340FOR I=1 TO N
350PRINT#B,D(I)
360NEXT
370PRINT"ENTER MORE DATA ?"
380GOTO160

```

**A.4. BBC BASIC programme to read body measurements,  
perform calculations and store results.**



```

10REM PROGRAMME C - FBAREAD
20REM READS BODY DATA AND PERFORMS CALCULATIONS.
30CLOSE£0
40MODE7
50DNE=99999
60*.
70INPUT"ENTER NAME OF FILE CONTAINING BODY
    DATA "BFILE$
80PROCGETNO
90DIM NUMB(Y%):DIM SLEN(Y%):DIM TLEN(Y%):
    DIM D(Y%,20):DIM RECT(Y%,20):
    DIM TRI(Y%,20)
100PROCGETBODY
110PROCWRITE
120PROCEND
130END
140
150DEFPROCGETNO
160Y%=1
170A=OPENUP("FA.OUT")
180REM GET SPP. DATE AND TANK n
190FOR S%=1 TO 3
200INPUT£A,A$
210NEXT
220REM GET NEXT STRING AND SEE IF END OF TANK *'s
    OR NEXT FISH
230INPUT£A,A$
240IF A$="*****" GOTO 190 ELSE GOTO 260
250REM GET MEAN AND SD VALUES FOR CF, DF, AND AF
260FOR X%=1 TO 6
270INPUT£A,T
280NEXT
290Y%=Y%+1
300E=EOF£A
310IF E=0 THEN GOTO220
320CLOSE£0
330ENDPROC
340
350DEFPROCGETBODY
360Y%=1:NUMB(0)=1
370B=OPENUP(BFILE$)
380INPUT£B,NUMB(Y%)
390INPUT£B,SLEN(Y%)
400INPUT£B,TLEN(Y%)
410LET CN=SLEN(Y%)/0.5
420IF CN<>NUMB(Y%) THEN EXTRA%=1 ELSE EXTRA%=0
430IF EXTRA%=1 AND CN>NUMB(Y%) THEN EXTRA%=2
440IF EXTRA%=1 THEN LESS=SLEN(Y%) -
    ((NUMB(Y%)-1)*0.5)
450IF EXTRA%=2 THEN PLUS=SLEN(Y%) -
    ((NUMB(Y%))*0.5)

```

```

460FOR S% = 1 TO NUMB(Y%)
470INPUT£B,D(Y%,S%)
480NEXT
490IF EXTRA%=2 THEN D(Y%,NUMB(Y%))=
      D(Y%,NUMB(Y%)-1)
500PROCCALCAREAS:PROCNUMBRESET
510E=EOF£B:IF E=0 Y%=Y%+1:GOTO 380
520CLOSE£0
530ENDPROC
540
550DEFPROCCALCAREAS
560 DIST=0.5
570FOR S% = 0 TO NUMB(Y%)-1
580 PROCAREAS
590NEXT
600 IF EXTRA%=2 THEN S%=NUMB(Y%):DIST=PLUS:
      D(Y%,S%+1)=D(Y%,S%):PROCAREAS
610 IF EXTRA%=1 THEN DIST=LESS:S%=NUMB(Y%)-1:
      PROCAREAS
620ENDPROC
630
640DEFPROCAREAS
650LET LONGER=0:LET SHORTER=0
660IF D(Y%,S%)>=D(Y%,S%+1) THEN
      SHORTER=D(Y%,S%+1)
670IF D(Y%,S%)>=D(Y%,S%+1) THEN LONGER=D(Y%,S%)
680IF D(Y%,S%)<D(Y%,S%+1) THEN SHORTER=D(Y%,S%)
690IF D(Y%,S%)<D(Y%,S%+1) THEN LONGER=D(Y%,S%+1)
700REM *** AREA RECTANGLE FIRST ***
710LET RECT(Y%,S%)=SHORTER*DIST
720REM *** AREA TRIANGLE NEXT ***
730LET TRI(Y%,S%)=(LONGER-SHORTER)*(DIST/2)
740ENDPROC
750
760DEFPROCWRITE
770C=OPENOUT("FA.OUT2")
780FOR X%=1 TO Y%
790PRINTNUMB(X%):PRINTSLEN(X%):PRINTTLEN(X%)
800PRINT£C,NUMB(X%),SLEN(X%),TLEN(X%)
810PRINT0:PRINT£C,0
820FOR S%=1 TO NUMB(X%)
830PRINTD(X%,S%);" ";RECT(X%,S%-1);" ";
      TRI(X%,S%-1)
840PRINT£C,D(X%,S%),RECT(X%,S%-1),TRI(X%,S%-1)
850NEXT
860PRINTDNE
870PRINT£C,DNE
880NEXT
890ENDPROC
900DEFPROCEND
910CHAIN"P.AREAS"

```

```
920ENDPROC
930
940DEFPROCNUMBRESET
950IF EXTRA%=2 THEN NUMB(Y%)=NUMB(Y%)+1
960ENDPROC
```

**A.5. BBC BASIC programme to produce a print-out of  
calculated surface area values and standard deviations.**

```

10REM PROGRAMME d - P.AREAS
20REM PRINTS OUT DATA SHEETS OF CALCULATED
   AREAS AND SD'S.
30MODE7
40CLOSE£0
50A=OPENUP ("FA.OUT")
60B=OPENUP ("FA.OUT2")
70PROCGET1
80PROCGET2
90PROCPRINTAREAS
100PROCDECIDENEXT
110GOTO90
120END
130
140DEFPROCGET1
150INPUT£A, SP$, D$, T$, F$
160ENDPROC
170
180DEFPROCGET2
190INPUT£A, MD, MC, MA, SD, SC, SA
200INPUT£B, N, SL, TL
210LET CN=SL/0.5
220ENDPROC
230
240DEFPROCPRINTAREAS
250VDU2
260PRINT''
270PRINT;STRING$(12," ");"Tank ";T$;
   "          Fish number : ";F$
280PRINT;STRING$(12," ");"Species      : ";SP$
290PRINT;STRING$(12," ");"Date        : ";D$
300PRINT;STRING$(12," ");"Total length = ";TL;
   "          Standard length = ";SL
310@%=£20305
320 PRINT'''
330PRINT"Distance";STRING$(6," ");"Body";
   STRING$(10," ");"Area";STRING$(6," ");
   "Area";STRING$(3," ");"Area";
   STRING$(4," ");"Culmulative"
340PRINT;STRING$(2," ");"from";STRING$(4," ");
   "circumferance";STRING$(2," ");"rectangle";
   STRING$(1," ");"triangle";STRING$(1," ");
   "sector";STRING$(4," ");"area"
350PRINT;" snout"
360PRINT" (cm)"
370PRINT':TT=0:L=0.5
380PRINTSTRING$(2," ");"0";STRING$(7," ");"0"
390Y%=0
400INPUT£B,D
410REPEAT
420INPUT£B,D

```

```

430IF D=99999.000 GOTO 480
440INPUT£B,R,T
450TT=TT+R+T
460PRINT " ";L;STRING$(7," ");D;STRING$(8," ");
      R;STRING$(5," ");T;STRING$(3," ");R+T;
      STRING$(6," ");TT
470L=L+0.5:IF L>SL L=SL
480REM
490UNTIL D=99999.000
500PRINT''
510PRINT"Area dorsal fin = ";MD;" SD= ";SD
520PRINT"Area caudal fin = ";MC;" SD= ";SC
530PRINT"Area anal fin = ";MA;" SD= ";SA
540@%=&20409
550PRINT'' :PRINT" Body surface area of one
      side = ";(TT/2)
560PRINT" Total fin area = ";MD+MC+MA
570PRINT'' :PRINT"Total surface area = ";
      (TT/2)+MD+MC+MA
580VDU3
590ENDPROC
600
610DEFPROCDECIDENEXT
620E1=EOF£A:E2=EOF£B
630IF E1=-1 OR E2=-1 THEN PROCEND
640INPUT£A,A$
650IF LEFT$(A$,4)="FISH" F$=A$:PROCGET2
660IF LEFT$(A$,4)="****" PROCGET1:PROCGET2
670ENDPROC
680
690DEFPROCEND
700VDU3
710PRINT"FA.OUT ----- EOF£ = ";E1
720PRINT"FA.OUT2 ---- EOF£ = ";E2
730CLOSE£0
740END
750ENDPROC

```

## Appendix B

**B.1. Isolates and strains of *Ichthyophthirius multifiliis* used in all experiments of this study.**

| Isolate   | Strain | Dates kept          | Number days kept | Comments   |
|-----------|--------|---------------------|------------------|--|
| Tropical  | A      | 16.2.85 - 6.8.85    | 171              | In later stages few experiments performed  |
| Tropical  | B      | 7.8.85              | ?                | Isolate never managed one passage  |
| Tropical  | C      | 13.9.85 - 4.11.85   | 52               | No successful experiments with this isolate  |
| Tropical  | D      | 11.11.85 - 25.11.85 | 14               | No successful experiments with this isolate  |
| Tropical  | E      | 25.11.85 - 31.12.85 | 36               |  |
| Tropical  | F      | 31.12.85            | ?                | Isolate never managed one passage  |
| Tropical  | G      | 13.1.86 - 13.6.86   | 151              | When lost some cysts did not develop and others produced small theronts                            |
| Tropical  | H      | 19.6.86 - 26.11.86  | 160              | Occasionally produced very low numbers of theronts, twice thought isolate lost and then reappeared |
| Temperate | I      | 10.3.87 - 30.4.87   | 51               | Levels of parasite very low in passage   |
| Tropical  | J      | 26.11.86 - 27.3.87  | 121              |  |
| Temperate | K      | 30.4.87 - 8.10.87   | 162              |  |
| Tropical  | L      | 27.3.87 - 4.12.87   | 252              |  |
| Tropical  | M      | 27.11.87 - 10.2.88  | 76               | In later stages very few theronts produced   |
| Tropical  | N      | 11.2.88 - 24.3.88   | 41               |  |
| Tropical  | O      | 7.4.88 - 15.5.88    | 38               |  |
| Tropical  | P      | 15.5.88 - 28.5.88   | 73               | This isolate kept by another worker simultaneously and also lost simultaneously                    |
| Tropical  | Q      | 14.6.88 - 10.7.88   | 26               | Never used in experiments - never produced large numbers of theronts                               |
| Tropical  | R      | 16.6.88 - 8.7.88    | 22               | Never used in experiments - never produced large numbers of theronts                               |
| Tropical  | S      | 18.6.88 - 10.7.88   | 22               | Never used in experiments - never produced large numbers of theronts                               |



## Appendix C

**C.1. Computer programme for heritability analysis and estimation, written in BASIC for a model B BBC microcomputer.**

```

10REM*****
20REM***** HERITABILITY ANOVA AND ESTIMATE *****
30REM***** CALCULATION FROM 'MANUAL OF *****
40REM***** QUANTITATIVE GENETICS by W.A. *****
50REM***** BECKER (1975) *****
60REM***** THIS VERSION DATED 5.2.88 *****
70REM***** ALSO INCLUDES CALCULATIONS FROM *****
80REM***** 'QUANTITATIVE GENETICS IN SHEEP *****
90REM***** BREEDING' by H.N. TURNER & *****
100REM***** S.S.Y. YOUNG (1969) *****
110REM***** @COPYRIGHT G.M. CLAYTON (1989) *****
120REM*****
130MODE7:@%=10
140*DR.2
150PRINT''''':PRINT"Enter new data (N) or load
old data (L)":REPEAT:A$=GET$:UNTIL A$="N"
OR A$="L":IF A$="N" PROCGETDATA ELSE
PROCRETDATA
160PROCsquareroot:PROCANOVA:PROCHERITABILITY:
PROCDICKERSON1959:PROCDISPLAY:PROCINVM:END
170
180DEFPROCGETDATA:INPUT"How many males ",NMALES%:
DIM NFEMALES(NMALES%):FOR X%=1 TO NMALES%:
PRINT';"How many females for male number ";
X%:INPUT""NFEMALES(X%):NEXT X%
190INPUT"What is the maximum number of offspring
for any female ",NOFFSPRING%:CLS:LET
TFEMALES%=0:FOR X%=1 TO NMALES%:TFEMALES%=
TFEMALES%+NFEMALES(X%):NEXT X%
200DIM offspring(TFEMALES%,NOFFSPRING%):CLS:TF%=0
210FOR NM%=1 TO NMALES%:NF%=NFEMALES(NM%):Q%=0:
FOR X%=(TF%+1) TO (TF%+NF%):Q%=Q%+1:PRINT
"Enter data for male ";NM%;" with female ";
Q%:NOFF%=0:REPEAT:NOFF%=NOFF%+1:INPUT""
offspring(X%,NOFF%):UNTIL offspring(X%,
NOFF%)=999 OR NOFF%>=NOFFSPRING%
220NEXT X%:TF%=TF%+NF%:NEXT NM%
230PRINT"Save data on disc Y/N ?":REPEAT:A$=GET$:
UNTIL A$="Y" OR A$="N":IF A$="N" ENDPROC
240INPUT"Enter filename "filename$:IF LEN
filename$>7 filename$=LEFT$(filename$,7)
250datafile=OPENOUT(filename%):PRINTfdatafile,
NMALES%,TFEMALES%,NOFFSPRING%
260FOR X%=1 TO TFEMALES%:FOR Y%=1 TO NOFFSPRING%:
PRINTfdatafile,offspring(X%,Y%):NEXTY%:NEXT
X%:FORX%=1 TO NMALES%:PRINTfdatafile,
NFEMALES(X%):NEXTX%
270CLOSEfdatafile:ENDPROC
280DEFPROCANOVA:REM SUM AND SUM OF SQUARES FIRST
290CLS:TF%=0:NOFF%=1:MOFF%=0:TLN%=0:DIM
SUM(TFEMALES%,2),FENOFF(TFEMALES%),

```

```

MNOFF (NMALES%)
300FOR NM%=1 TO NMALES%:NF%=NFEMALES (NM%):
  FOR X%=(TF%+1) TO (TF%+NF%):SUM=0:SS=0:
  REPEAT
310IF offspring(X%,NOFF%)<>999 SUM=SUM+offspring
  (X%,NOFF%):MOFF%=MOFF%+1
320IF offspring(X%,NOFF%)<>999 SS=SS+(offspring
  (X%,NOFF%)*offspring(X%,NOFF%)):IF NOFF%<
  NOFFSPRING% NOFF%=NOFF%+1:GOTO 310
330UNTIL (NOFF%=NOFFSPRING%) OR (offspring(X%,
  NOFF%)=999):IF NOFF%<=NOFFSPRING% AND
  offspring(X%,NOFF%)=999 NOFF%=NOFF%-1
340SUM(X%,1)=SUM:SUM(X%,2)=SS:FENOFF(X%)=NOFF%:
  TLN%=TLN%+NOFF%:NOFF%=1:NEXT X%:TF%=TF%+
  NF%:MNOFF(NM%)=MOFF%:MOFF%=0:NEXT NM%
350REM Compute grand total of Y and Y squared
360Y1=0:Y2=0:FOR X%=1 TO TFEMALES%:Y1=Y1+
  SUM(X%,1):Y2=Y2+SUM(X%,2):NEXT X%
370REM Compute Correction Term
380CT=(Y1*Y1)/TLN%
390REM Calculate between sires SS and MS
400TF%=0:TY2S=0:FOR NM%=1 TO NMALES%:NF%=
  NFEMALES (NM%):SUM=0:FOR X%=(TF%+1)
  TO (TF%+NF%):SUM=SUM+SUM(X%,1):NEXT
  X%:TY2S=((SUM*SUM)/MNOFF(NM%))+TY2S:
  TF%=TF%+NF%:NEXT NM%:SSBS=TY2S-CT:
  MSBS=SSBS/(NMALES%-1)
410REM Calculate between dams SS and MS
420TY2D=0:TY2P=0:FOR X%=1 TO TFEMALES%:TY2D
  =TY2D+((SUM(X%,1)*SUM(X%,1))/FENOFF(X%)):
  TY2P=TY2P+SUM(X%,2):NEXT X%:SSBD=TY2D-
  TY2S:MSBD=SSBD/(TFEMALES%-NMALES%)
430REM Calculate between progeny within dams
  SS and MS
440SSBP=Y2-TY2D:MSBP=SSBP/(TLN%-TFEMALES%):
  ENDPROC
450DEFPROCDISPLAY:J$="":PRINT'"Do you want a
  print-out Y/N?":REPEAT:J$=GET$:UNTIL
  J$="Y" OR J$="N":CLS:IF J$="Y"VDU2
460@%=&20809:PRINT STRING$(24," ");filename$:
  PRINT'"Analysis of variance.":PRINTSRTE$:
  PRINT'"Source of variation";SPC17;"d.f.";
  SPC14;"SS";SPC12;"MS"
470PRINT'"Between Sires";SPC21;NMALES%-1;SPC2;
  SSBS;SPC2;MSBS:PRINT'"Between Dams,
  within Sires";SPC8;TFEMALES%-NMALES%;SPC2;
  SSBD;SPC2;MSBD:PRINT'"Between Progeny,
  within Dams";SPC6;TLN%-TFEMALES%;SPC2;
  SSBP;SPC2;MSBP
480GAP$=STRING$(9," "):PRINT'"Estimates from
  Becker (1975), Turner & Young (1969) and

```

```

Dickerson (1959) "
490PRINT' 'GAP$;"K1 = ";K1:PRINTGAP$;"K2 = ";K2:
PRINTGAP$;"K3 = ";K3:PRINT' :PRINTGAP$;"Ow
= ";W2:PRINTGAP$;"Od = ";D2:PRINTGAP$;"Os
= ";S2:PRINT' :PRINTGAP$;"hs = ";H2S;" S.E.
= ";SEH2S:PRINTGAP$;"hd = ";H2D;" S.E. = ";
SEH2D:PRINTGAP$;"hsd = ";H2SD;" S.E. = ";
SEH2SD
500PRINT' :VDU3:ENDPROC
510DEFPROCHERITABILITY:REM HERITABILITY
CALCULATIONS ACCORDING TO BECKER (1975)
520REM Calculate K1
530K=0:TF%=0:K1=0
540FOR NM%=1 TO NMALES%:NF%=NFEMALES(NM%):K=0:
FOR X%=(TF%+1) TO (NF%+TF%):K=K+(FENOFF
(X%)*FENOFF(X%)):NEXT X%:K1=K1+(K/MNOFF
(NM%)):TF%=TF%+NF%:NEXT NM%:PS=K1:K1=
(TLN%-K1)/(TFEMALES%-NMALES%)
550REM Calculate K2
560K=0:K2=0
570FOR X%=1 TO TFEMALES%:K=K+(FENOFF(X%)*FENOFF
(X%)):NEXT X%:K=K/TLN%:K=PS-K:K2=K/
(NMALES%-1)
580REM Calculate K3
590K=0:K3=0
600FOR X%=1 TO NMALES%:K=K+(MNOFF(X%)*MNOFF
(X%)):NEXT X%:K3=(TLN%-(K/TLN%))/
(NMALES%-1)
610REM Calculate ow, od, os, op
620W2=MSBP:D2=(MSBD-MSBP)/K1:S2=(MSBS-MSBP-
((K2/K1)*(MSBD-MSBP)))/K3:VP=S2+D2+W2
630REM Calculate SE's of s2 d2 and w2
640SES2=(MSBS^2)/(NMALES%-1+2):SES2=SES2+
((MSBD^2)/((TFEMALES%-NMALES%)+2)):
SES2=SES2*(2/(K3^2)):SES2=SQR(SES2)
650REM Calculate Heritabilities
660H2S=(4*S2)/(VP):H2D=(4*D2)/(VP):H2SD=
(2*(S2+D2))/(VP):ENDPROC
670DEFPROCRETDATA:REM OBTAINS DATA FROM
ALREADY CREATED FILE
680PRINT' ':*
690PRINT':INPUT"Enter filename of data file "
filename$:IF LEN filename$>7 filename$=
LEFT$(filename$,7)
700datafile=OPENUP(filename$):INPUTfdatafile,
NMALES%,TFEMALES%,NOFFSPRING%:DIMoffspring
(TFEMALES%,NOFFSPRING%),NFEMALES(NMALES%)
710FOR X%=1 TO TFEMALES%:FOR Y%=1 TO NOFFSPRING%:
INPUTfdatafile,offspring(X%,Y%):NEXTY%:NEXT
X%:FORX%=1 TO NMALES%:INPUTfdatafile,
NFEMALES(X%):NEXTX%:CLOSEfdatafile:ENDPROC

```

```

720DEFPROC DICKERSON1959
730REM ESTIMATES OF SE'S FOR HERITABILITIES
      TAKEN FROM p111 OF TURNER & YOUNG
740REM (1969) "GENETICS IN SHEEP BREEDING",
      ACCORDING TO ESTIMATION BY
750REM DICKERSON (1959).
760DK1=K2:DK2=K3:DK3=K1
770A=SQR((2/(DK2^2))*(((MSBS^2)/(NMALES%-1))
      +((MSBD^2)/(TFEMALES%-NMALES%)))):B=SQR
      ((2/(DK3^2))*(((MSBD^2)/(TFEMALES%-
      NMALES%))+((MSBP^2)/(TLN%-TFEMALES%))))):
      C=(-1*(DK1/DK2))*((B^2)-((2*(MSBP^2))/
      ((DK3^2)*(TLN%-TFEMALES%))))
780REM CALCULATE HERITABILITY SE's
790SEH2S=SQR((4*A)/VP):SEH2D=SQR((4*B)/VP):
      SEH2SD=((A^2)+(B^2)+(C^2)):IF SEH2SD<0
      THEN SEH2SD=0:ENDPROC:REM CANNOT SQR
      -ve NUMBER.
800SEH2SD=SQR((2*SQR(SEH2SD))/VP):ENDPROC
810DEFPROC squareroot:SRTF$=STRING$(30," "):
      PRINT' "Do wish to perform
      1 A square root transformation.
      2 A Ln transformation.
      3 A Log transformation.
      4 No transformation."
820REPEAT:SR$=GET$:UNTIL SR$="1" OR SR$="2" OR
      SR$="3" OR SR$="4":IF SR$="4" ENDPROC
830 IF SR$="1" GOTO 860
840 IF SR$="2" GOTO 880
850 IF SR$="3" GOTO 900
860FOR X%=1 TO TFEMALES%:FOR Y%=1 TO NOFFSPRING%:
      IF offspring(X%,Y%)<> 999 AND offspring
      (X%,Y%)<> 0 THEN offspring(X%,Y%)=
      SQR(offspring(X%,Y%))
870NEXT:NEXT:SRTF$="Square root transformed
      data":ENDPROC
880IF SR$<>"2" STOP ELSE FOR X%=1 TO TFEMALES%:
      FOR Y%=1 TO NOFFSPRING%:IF offspring
      (X%,Y%)<>999 AND offspring(X%,Y%)<> 0
      THEN offspring(X%,Y%)=LN(offspring(X%,Y%))
890NEXT:NEXT:SRTF$="Ln transformed data":ENDPROC
900IF SR$<>"3" STOP ELSE FOR X%=1 TO TFEMALES%:
      FOR Y%=1 TO NOFFSPRING%:IF offspring(X%,Y%)
      <> 999 AND offspring(X%,Y%)<> 0 THEN
      offspring(X%,Y%)=LOG(offspring(X%,Y%)):
      NEXT:NEXT:SRTF$="Log transformed data":
      ENDPROC
910
920
930
940*****

```

```

950DEFPROCINVM
960M=3
970REM FINDS INVERSE OF 4x4 MATRIX
980@%=&20406
990DIM I(M,M),M(M,M),MS(M),g2(M),C(M,M),
      Num(M-1),Denom(M-1),F(M-1)
1000FOR X=1 TO M:I(X,X)=1:NEXT
1010FOR X=1 TO 3:M(3,X)=1:NEXT
1020M(1,1)=K3:M(2,1)=K2:M(2,2)=K1
1030C(1,1)=K3:C(2,1)=K2:C(2,2)=K1:
      FOR X=1 TO 3:C(3,X)=1:NEXT
1040MS(1)=MSBS:MS(2)=MSBD:MS(3)=MSBP
1050PROCD
1060A$=GET$
1070FOR S=1 TO M
1080FOR X=1 TO M:IF M(S,X)<0 PROCMINUS(X)
1090NEXT
1100FOR T=1 TO M
1110IF S=T GOTO 1150
1120IF M(S,T)>0 F=M(S,S)/M(S,T) ELSE GOTO 1140
1130PROCCALC(T,S)
1140CLS:PROCD
1150NEXT T
1160NEXT S
1170PROCONE
1180CLS:PROCD
1190PROCF
1200PROCEND:ENDPROC
1210DEFPROCDF:FOR Y=1 TO M:FOR X=1 TO M:
      PRINTI(X,Y);" ";:NEXT:PRINT:NEXT:
      PRINT''':FOR Y=1 TO M:FOR X=1 TO M:
      PRINTM(X,Y);" ";:NEXT:PRINT:NEXT:
      ENDPROC
1220DEFPROCDFMINUS(Z)
1230FOR Q=1 TO M:M(Q,Z)=M(Q,Z)*-1:I(Q,Z)=
      I(Q,Z)*-1:NEXT
1240ENDPROC
1250DEFPROCDFCALC(Z,Q)
1260FOR X=1 TO M:M(X,Z)=M(X,Z)*F:I(X,Z)=
      I(X,Z)*F:NEXT
1270FOR X=1 TO M:I(X,Z)=I(X,Z)-I(X,Q):
      M(X,Z)=M(X,Z)-M(X,Q):NEXT
1280FOR Y=1 TO M:FOR X=1 TO M:IF M(X,Y)
      <0.000001 AND M(X,Y)>0 M(X,Y)=0
1290IF I(X,Y)<0.000001 AND I(X,Y)>0 I(X,Y)=0
1300NEXT:NEXT
1310ENDPROC
1320DEFPROCDFCONE
1330FOR Y=1 TO M
1340F=M(Y,Y)
1350FOR X=1 TO M

```

```

1360M(X,Y)=M(X,Y)/F:I(X,Y)=I(X,Y)/F
1370NEXT: NEXT:ENDPROC
1380DEFPROCEND
1390PRINT"DO YOU WANT A PRINT OUT Y/N":
      P$=GET$:IF P$="Y" CLS:VDU2:PROCDF:
      PRINT'':PROCFP:VDU3:END
1400END
1410ENDPROC
1420DEFPROCF
1430FOR X=1 TO M:FOR Y=1 TO M:g2(X)=(I(Y,X)
      *MS(Y))+g2(X):NEXT:NEXT
1440FOR X=1 TO M-1
1450Num(X)=MS(X)
1460FOR Y=X+1 TO M
1470Denom(X)=Denom(X)+(g2(Y)*C(Y,X)):NEXT Y
1480F(X)=Num(X)/Denom(X)
1490NEXT X
1500ENDPROC
1510DEFPROCFP:PROCdenomdf:PRINT
      "Num      Denom      F      df(N)      df(D)      P"
1520PRINTNum(1);" ";Denom(1);" ";F(1);
      " ";(NMALES%-1);" ";DDFS2:PRINTNum(2);
      " ";Denom(2);" ";F(2);" ";(TFEMALES%-
      NMALES%);" ";DDFD2
1530ENDPROC
1540
1550DEFPROCdenomdf
1560DDFS2=(K3*I(2,1)*MSBD)^2/(TFEMALES%-NMALES%)
1570DDFS2=DDFS2+(K3*I(3,1)*MSBP)^2/
      (TLN%-TFEMALES%)
1580DDFS2=(Denom(1)^2)/DDFS2
1590DDFD2=(K1*I(3,2)*MSBP)^2/(TLN%-TFEMALES%)
1600DDFD2=(MSBP^2)/DDFD2
1610ENDPROC

```



## Appendix D

## **D.1. List of publications and conference papers given as a result of this study.**

Clayton, G.M. & Price, D.J. (1987) Genetic variation to *Ichthyophthirius multifiliis* infections. **European Association of Fish Pathologists Third International Conference, Bergen, Norway, August 31<sup>st</sup>- September 3<sup>rd</sup>.**

Clayton, G.M. & Price, D.J. (1987) *Ichthyophthirius multifiliis* infection in carp (*Cyprinus carpio* L.): genetic variation in susceptibility. **Aquaculture Europe International Conference and Exhibition, Amsterdam, Holland, June 2<sup>nd</sup>-5<sup>th</sup>.**

Clayton, G.M. & Price, D.J. (1988) *Ichthyophthirius multifiliis*: standardization of the infection-response model in *Ameioba splendens* (Miller & Fitzsimons). *Journal of Fish Diseases*, 11, 371-377.

Clayton, G.M. & Price, D.J. (1988) Interspecific and intraspecific variation in resistance to white spot in teleost fish. **Population Genetics Group, London, January 6<sup>th</sup>-8<sup>th</sup>.**

Clayton, G.M. & Price, D.J. (1987) Pleiotropic effects of scale pattern genes in common carp: susceptibility to *Ichthyophthirius multifiliis* infection. **Genetical Society, London, November 13<sup>th</sup>-14<sup>th</sup>.**

Clayton, G.M. & Price, D.J. (1987) Standardization of infection and response to white spot (*Ichthyophthirius multifiliis*) in fish. **Fisheries Society of the British Isles Immunology Symposium, Plymouth Polytechnic, July 20<sup>th</sup>-24<sup>th</sup>.**

Clayton, G.M. & Price, D.J. (1987) Standardization of infection and response to white spot, *Ichthyophthirius multifiliis*. *Journal of Fish Biology*, 31 (Supplement A), 241-242.